The minimal Tat translocase

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A two-residue peptide, consisting of two arginine amino acids displayed in a scaled ball and stick model.

RIJKSUNIVERSITEIT GRONINGEN

The minimal Tat translocase

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Promotor:	Prof. dr. J.M. van Dijl
Copromotor:	dr. J.-Y. F. Dubois
Beoordelingscommissie**:**	Prof. dr. A. J. Driessen Prof. dr. W. J. Quax Prof. dr. T. Palmer

Paranimfen: Mark J.J.B. Sibbald
Thijs R.H.M. Kouwen

The voyage of discovery is not in seeking new landscapes but in having new eyes.

-Marcel Proust-

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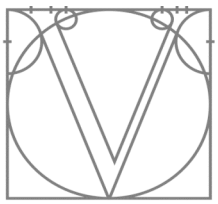

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GUIDE

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Chapter 1

Introduction to protein secretion

COMPARTMENTALIZATION

Life on earth started almost 4 billion years ago. How life exactly emerged is a matter of continuous debate and speculation. However, by definition, the first living cell must have maintained a protected internal compartment that was probably enclosed by lipids. Continuous cycles of growth, replication of nucleic acids, and cell division were subsequently needed to sustain life. This imposed even on the first living cells a requirement for uptake of nutrients and disposal of waste products, making transport of the respective molecules across the lipid membrane necessary. During time sophisticated transport systems and channels have evolved to perform these and other more specific functions. Cells as we know them today employ membrane transport for a wide range of different processes, including maintenance of constant internal ion concentrations that can differ substantially from the concentrations of the respective ions in the environment, transport of nutrients into the cytoplasm (*e.g.* carbohydrates, amino acids, peptides and lipids), regulation of metabolite concentrations, extrusion of (toxic) waste compounds, transport of nucleic acids for the exchange of genetic information, and the secretion of pheromones, toxins, and enzymes. All these transport processes serve to increase the cell's chances of survival in sometimes very unfavourable environments. Since all the processes rely on membrane-embedded proteinaceous channels and often involve exported proteins, it is evident that protein transport processes from the cytoplasm to the membrane and beyond are of the utmost importance for cellular survival and competitive success in different ecological niches.

BACILLUS SUBTILIS

The soil contains many different niches where microorganisms can thrive on dead organic matter. This usually requires the high-level secretion of degradative enzymes for nutrient provision of the cells. A well-known organism that can be found in the soil and plant rhizosphere is *Bacillus subtilis*. *B. subtilis* is a Gram-positive rod-shaped bacterium with a thick cell wall. Although generally regarded as a strictly aerobic organism, *B. subtilis* can also display anaerobic growth on nitrate [1]. For competitive success in its natural niches, *B. subtilis* applies a range of different survival strategies, including motility, competence development, synthesis of antibiotics and sporulation. Genetic competence for the uptake and integration of DNA allows the

cells to evolve and adapt through horizontal gene transfer [2]. Synthesis of a flagellum allows *B. subtilis* to reach the most favourable milieus for growth [3,4]. Antibiotics are produced for the defence against competing bacteria [5,6]. Life-threatening, harsh and severely nutrient-limited environmental conditions are overcome by producing spores for long-term survival [7].

During vegetative growth, nutrients are liberated from the dead organic matter in the environment through a wide range of enzymatic reactions catalyzed by the different enzymes secreted by *B. subtilis*. These enzymes include, for example, proteases, lipases and alpha-amylases that are actually of high commercial interest due to a wide range of industrial and consumer applications [8]. Importantly, the cell envelope of *B. subtilis*, being a Gram-positive organism, includes just one membrane. Thus, proteins that are transported across the membrane can be directly transferred to the environment, provided that they do not contain signals for membrane- or cell wall retention. As a consequence, huge extracellular protein concentrations of up to 20 g /l can be reached. Secreted protein concentrations of more than 1 g/l are usually considered commercially significant, and this high potential for protein secretion is actually one of the prime reasons why *B. subtilis* has been developed into a major industrial workhorse. Other good reasons to apply *B. subtilis* in the biotechnology industry are its Generally Regarded As Safe (GRAS) status and the ease of genetic engineering due to the natural development of genetic competence. In turn, the applied interest in protein secretion by *B. subtilis* has boosted fundamental studies on protein secretion mechanisms in this organism. Thus, *B. subtilis* has become the paradigm for studies on protein secretion in Gram-positive bacteria in general. Clearly, this general knowledge about protein secretion feeds back into applications of *Bacillus* for production of secreted proteins, including enzymes and biopharmaceuticals. A possible future spin-off of the *Bacillus* secretion research is the identification of novel potential targets for drugs, vaccines or therapeutic antibodies in important Gram-positive pathogens like *Bacillus anthracis*, *Bacillus cereus*, *Listeria monocytogenes*, or *Staphylococcus aureus* [9].

PROTEIN SECRETION

The bacterial cell is a highly dynamic system continuously searching for optimal growth conditions. During every step of its life cycle, protein export from the cytoplasm plays an important role in numerous growth-determining processes. The export of proteins requires dedicated transport machineries, which are usually composed of channels and motor proteins that use the energy provided through ATP hydrolysis and/or the proton-motive force to drive protein movement across the membrane. Evolutionary related protein transport systems are found in Gram-positive and Gram-negative bacteria. Gram-positive bacteria, which have only one membrane, usually secrete their proteins into the external milieu. By contrast, Gram-negative bacteria use the homologous protein transport systems generally for protein export to the periplasm between the inner and outer membranes, Figure 1. In the latter group of organisms, some translocated proteins are subsequently fed into dedicated pathways for passage across the outer membrane [10].

Proteins are synthesized in the cytoplasm of bacterial cells but, due to the action of membrane translocation and sorting machineries, some proteins will end up at different extracytoplasmic locations of the cell. Computer-assisted predictions indicate that about 25% of the proteins of a bacterial cell have amino-terminal signal peptides that serve to guide these proteins to an extracytoplasmic destination [11,12]. For *B. subtilis* at least five different extracytoplasmic destinations can be distinguished [12]. These are the membrane lipid bilayer, the membrane-cell wall interface, the cell wall, the extracellular milieu, and the intermembrane space between forespore and mother cell that is temporarily created during sporulation. In principle, the exported proteins will travel to the extracellular milieu unless they are provided with a specific retention signal, or they are transported into the intermembrane space between a developing forespore and its mother cell. Retention at the membrane-cell wall interface can be facilitated by a hydrophobic stretch of amino acids that form a transmembrane anchor. Alternatively, translocated proteins are retained at the extracytoplasmic membrane surface through diacyl-glyceryl modification at a so-called lipobox [13-15]. Cell wall retention can be facilitated by cell wall binding domains, covalent attachment to the cell wall due to the action of sortases, or electrostatic interactions with cell wall components [16,17]. It should be noted that this cell wall retention is usually not 100% effective. Notably, about 25% of the detectable extracellular proteins lack a predictable secretion signal. Such proteins may be liberated from the cells through lysis, export via the flagellar assembly

machinery or holins, or so far unidentified alternative pathways [13]. Clearly, the so far best characterised systems for active protein transport across the cytoplasmic membrane are the general Secretion (Sec) pathway and the Twin-arginine translocation (Tat) pathway. The majority of extracytoplasmic *B. subtilis* proteins seem to be exported via the Sec pathway [12]. Interestingly, a relatively large number of proteins was predicted to follow the Tat pathway in early secretome predictions, but subsequent proteomics analyses and directed studies revealed that most of these proteins were nevertheless secreted via the Sec pathway [18,19]. This unexplained observation and the fact that the Tat pathway has an intrinsic potential to transport folded proteins, in contrast to the Sec pathway, focused attention on the Tat pathway of *B. subtilis*. The *B. subtilis* Tat pathway for protein export from the cytoplasm is therefore the main subject of the studies described in this thesis.

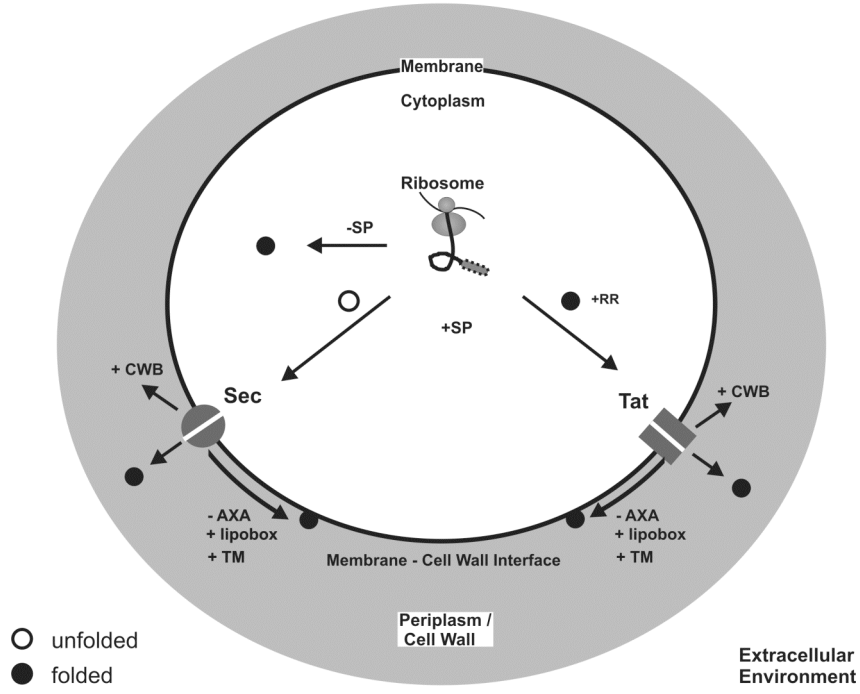

Figure 1. Secretion via the Sec and Tat pathway. RR, twin-arginine signal peptide; SP, general signal peptide; CWB, cell wall binding proteins; AXA, type I signal peptidase cleavage site; Lipobox, lipobox for lipid modification of membrane-attached lipoproteins; TM, transmembrane anchor

PROTEIN SECRETION PATHWAYS

The Sec pathway for protein transport is ubiquitously conserved in prokaryotes, while the Tat pathway is present in many, but certainly not all, prokaryotes. The Sec pathway is in fact conserved in all three kingdoms of life [20]. It is essential for cell growth and viability, as it facilitates export of the majority of extracytoplasmic proteins. The composition and mode-of-action of the Sec translocase have been studied to great detail. In recent years, high-resolution crystal structures of the components has provided valuable information on the transport mechanism [21]. The bacterial Sec pathway moves proteins in an unfolded state across the membrane, like a thread through a needle, employing a pushing mechanism [22]. In the cytoplasm the transported protein is kept unfolded due to the activity of chaperones, such as the *Escherichia coli* SecB protein [23]. Complexes of secretory precursor proteins and chaperones are delivered to SecA, the motor protein of the Sec translocation machinery. Once accepted by the Sec machinery, the precursor protein is pushed through the SecYEG channel in a process that requires repeated cycles of ATP-binding and hydrolysis by SecA and the proton-motive force [24-26]. It should be noted that the Sec pathway seems completely unable to facilitate the transport of folded proteins across the lipid bilayer [19]. On the *trans* side of the membrane, proteins translocated via Sec need to be liberated from the membrane and to fold into their native and biologically active conformation. These processes require the action of signal peptidases for signal peptide removal, folding catalysts (*e.g.* peptidyl-prolyl cis/trans isomerases), enzymes for post-translocational modifications (*e.g.* thiol-disulfide oxidoreductases for disulfide bond formation), and chaperones for the formation of quaternary structures (e.g. Oxa1p-like proteins) [27].

The Tat pathway is present in prokaryotes and chloroplasts of green plants. Recent data suggest that a few archaea actually use the Tat pathway as the major route for protein export from the cytoplasm [28-31]. The Tat pathway works independently from the Sec pathway and employs a completely different mechanism. Although, a few unfolded proteins can be transported via Tat, it is generally accepted that the main function of Tat is exporting folded proteins [32]. In general, the energy for Tat-dependent protein transport is provided by the ΔpH component of the proton-motive force (pmf). However, it was recently shown that halophilic archaea apply a sodium motive force to drive Tat-dependent protein transport [33]. The Tat pathway is appreciated for its ability to transport folded globular proteins, proteins

with bound co-factors and even protein complexes [34-38]. The existence of this pathway was first discovered in the thylakoids of chloroplasts, where protein transport was inhibited when the proton motive force was absent [39,40]. Therefore the Tat pathway was initially called the ΔpH-dependent pathway. Based on the results obtained with chloroplasts, the presence of Tat pathways was predicted in bacteria before it was actually shown to be functional [41]. Proteins transported across the thylakoid membrane via this pathway were found to contain a sequence motif with two consecutive (twin) arginines located in the N-terminus of their signal peptides [42]. Bacterial twin-arginine signal peptides were able to direct thylakoidal transport via the ΔpH-dependent pathway pointing towards the existence of this pathway in bacteria [43]. Two components of the thylakoidal Tat pathway were identified through the characterization of gene mutations that resulted in an inability to transport ΔpH-dependent proteins into the lumen of thylakoids [44,45]. Homologous gene products were subsequently identified in bacteria with most of the pioneering work being performed in *E. coli* [46-48]. In this Gram-negative bacterium, four important Tat proteins are encoded by an operon of four genes named *tatA, tatB, tatC,* and *tatD* (*tatABCD*). A fifth gene, *tatE*, is located on another position of the chromosome (Fig. 2). The TatABC components are essential for protein translocation activity, whereas TatE is dispensable [49,50]. No specific function in protein translocation has so far been assigned to TatE. The three structurally related proteins TatA, TatB and TatE have a similar topology containing a single transmembrane region with an N-terminal region facing the extracytoplasmic membrane surface and a C-terminal cytosolic amphipathic region (Fig. 3). The highest similarity is detectable between the TatA and TatE proteins. [51,52]. TatE is able to restore protein export in the absence of TatA, but clearly with a lower efficiency [53].

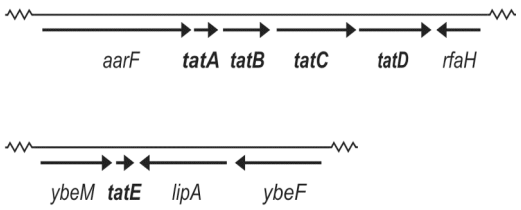

Figure 2. The *tat* genes present in *E. coli*.
Found at two different loci in the genome.

The TatD protein was originally described as a protein with DNAse activity that is not involved in the translocation process. However, very recently it was reported that TatD has a role in the quality control of secreted Tat-dependent proteins [54,55].
Phylogenetic analyses using annotated genome sequences revealed that homologues of the thylakoidal and *E. coli* Tat proteins are present in many but not all prokaryotes [28]. Also, differing numbers

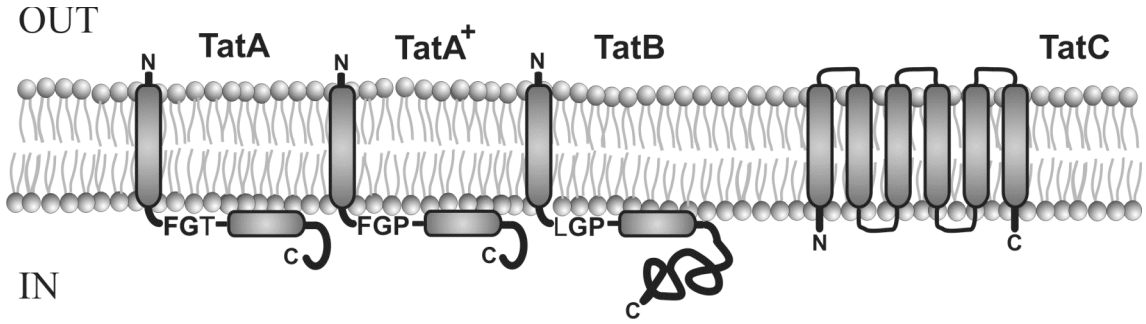

Figure 3. Topology of Tat pathway components. TatA and TatA$^+$ ($^+$ bifunctional TatA) proteins, TatB and TatC proteins

of Tat proteins were identified, indicating differences in the composition of the Tat translocation pathways in different prokaryotes. Gram-positive bacteria appeared to have only two proteins to make a functional Tat pathway, a TatA and a TatC subunit. In view of the structural similarity between *E. coli* TatA and TatB, this suggested that the TatA protein of Gram-positive bacteria is bifunctional, simultaneously performing the functions of *E. coli* TatA and TatB. [56]. Due to its limited complexity, the Gram-positive bacterial TatAC type system can thus be regarded as the minimal Tat system [57]. The idea that the Gram-positive TatA subunits are bifunctional was recently confirmed by heterologous expression of the TatAd protein from *B. subtilis* in *E. coli*, which showed that TatAd can functionally replace the absence of either TatA or TatB [58].

The mechanism of Tat-facilitated protein translocation is the subject of extensive ongoing research. The current consensus of opinion is that the TatC subunit, together with TatA$^+$ or TatB (TatA$^+$ indicates the bifunctional TatA from Gram-positive bacteria) forms a signal peptide receptor with a 1:1 subunit stochiometry [59,60]. This receptor initially interacts with the twin-arginine motif in the signal peptide, which then leads to a conformational change or exposes a binding site that allows the binding of multiple complexed TatA proteins, Figure 4 [59]. The TatA proteins will then form a channel for passage of the Tat-dependent secretory protein.

The *E. coli* TatA protein can form complexes of different sizes that may facilitate the formation of flexible pores, which would allow the passage of proteins with widely varying diameters [61]. Interestingly, the *B. subtilis* TatAdCd system forms a more homogeneous complex than *E. coli* TatABC, suggesting that TatAdCd has less flexible channel dimensions. This might point towards a functional difference between the TatABC

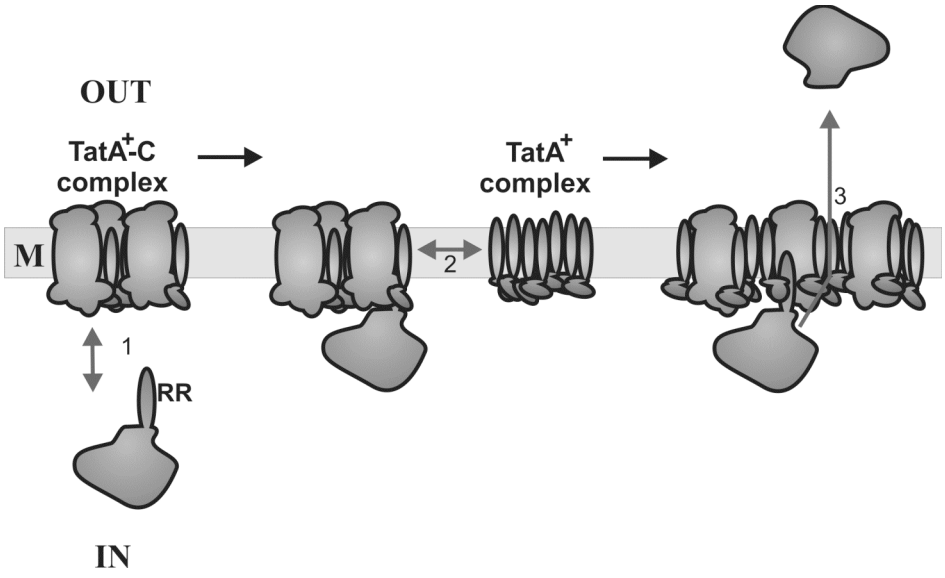

Figure 4. The general steps in the translocation process for the minimal Tat translocases. At step 1, the $TatA^{+}C$ receptor recognizes and binds the Tat signal peptide; step 2, channel formation together with $TatA^{+}$ protomers and step 3, protein translocation with signal peptide cleavage.

and TatAC systems [58]. During or shortly after precursor translocation, the signal peptide is cleaved and the Tat-precursor complex disassembles. How the energy of the ΔpH drives protein transport via Tat is currently unresolved, but the ΔpH seems not to be essential for the formation of Tat complexes. The energy consumption for Tat-dependent protein translocation has been established particularly well in thylakoids [62].

The data published so far suggest that the Tat systems of certain organisms may have evolved specific features. For example, TatAd was identified not only as a membrane protein of *B. subtilis*, but also as a soluble protein that has the potential to interact with the PhoD precursor protein. This precursor specifically requires the minimal TatAdCd translocase for export, which suggests that the soluble TatAd may have a chaperone-like function in guiding the precursor to the translocase [63]. Intriguingly, a soluble TatA form has been observed also in other bacteria, such as *Streptomyces* [64]. In chloroplasts, a pool of soluble TatA proteins seems able to reconstitute translocation across the thylakoidal membrane if insufficient membrane-localized TatA is present [65]. Furthermore, some halophilic archaea contain a TatAC type Tat system similar to the Gram-positive bacteria, but with an interesting difference: in these organisms, a TatC subunit is present that has twice the size of a normal TatC protein [30]. Furthermore, in these halophilic archaea the Tat pathway seems responsible for secretion of the majority of the secretory proteins thereby making it indispensable for

viability [66]. Different from the Tat pathways in most other organisms, the Tat pathway of halophilic archaea was shown to employ a sodium motive force instead of the pmf as an energy requirement [33]. Possibly, the large TatC subunit has evolved to facilitate the usage of a sodium-motive force for driving protein transport. However, this remains to be shown. In any case, the available data suggest that the Tat pathways in halophilic archaea are substantially different from the bacterial Tat pathways.

Despite differences in subunit composition and particular properties of individual Tat components, Tat deficient strains can often be complemented by heterologously expressed Tat systems [67,68]. For example, the *E. coli* TatABC translocase, which has an average size of 600 kDa, can be replaced with TatABC systems of the Gram-negative bacteria *Agrobacterium tumefaciens* and *Salmonella typhimurium.* Functional complementation for translocation of the 90-kDa TMAO reductase (TorA) was even achieved with the *B. subtilis* TatAdCd translocase, which has a significantly smaller size (~350 kDa) than the *E. coli* TatABC translocase [58]. Thus, it seems that Tat function has been conserved in evolution, despite the substantial differences that have emerged in individual Tat components and complexes.

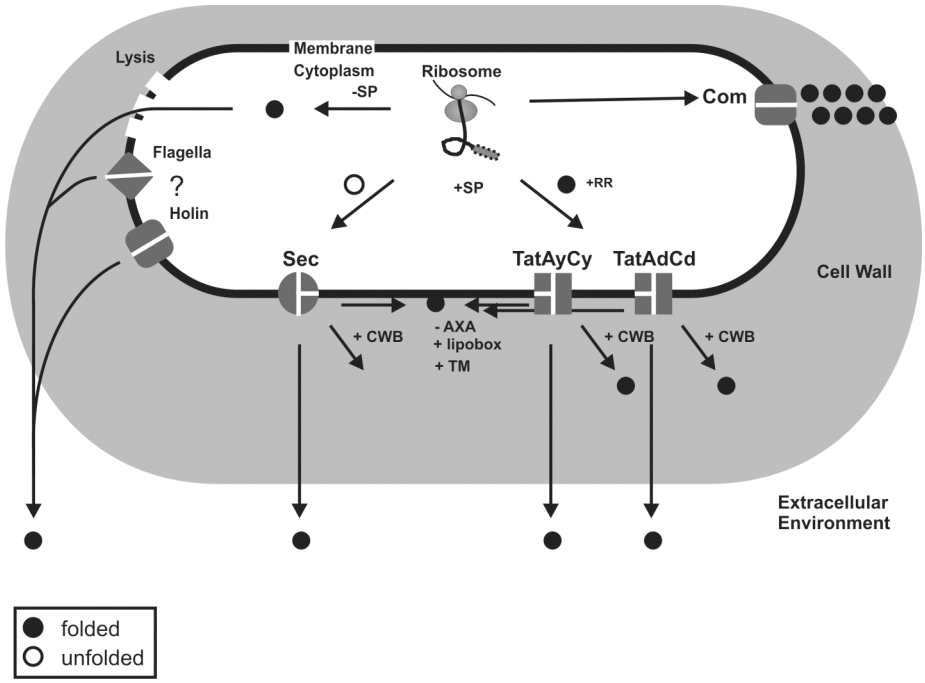

Figure 5. **Pathways of secretion present in *B. subtilis*.** RR, twin-arginine signal peptide; SP, general signal peptide; CWB, cell wall binding proteins; AXA, type I signal peptidase cleavage site; Lipobox, lipobox for lipid modification of membrane-attached lipoproteins; TM, transmembrane anchor

THE MINIMAL TAT PATHWAY OF *B. SUBTILIS*

Several protein transport pathways exist in *B. subtilis* that direct proteins to extracytoplasmic cellular locations or the growth medium. However, not all the proteins found in the extracellular milieu can be correlated to the activity of a particular transport system through directed analyses, proteomics or signal peptide-based predictions. Some proteins might actually reach the external milieu due to cell lysis. The main protein export route is the Sec pathway. In addition, proteins can leave the cytoplasm via the Tat pathway, holins, or the flagellar assembly machinery. Like several other species, *B. subtilis* has actually two Tat translocases that can work in parallel. These two translocases have been named TatAyCy and TatAdCd [69]. In addition to the two respective *tatA* genes (*i.e. tatAy* and *tatAd*), the genome of *B. subtilis* encodes a third *tatA* gene named *tatAc*. Downstream of *tatAy* and *tatAd*, *tatC* genes are located which have been named *tatCy* and *tatCd*, respectively (Fig. 6). No *tatC* counterpart has been identified for *tatAc*. The currently available data suggest that *tatAc* is a duplicated gene that is completely dispensable for Tat-dependent protein translocation. Furthermore, the *tatAc* gene is unable to complement for the absence of *tatAy* or *tatAd* [70].

Although, the respective TatA and TatC subunits of the two *B. subtilis* Tat systems show strong similarities, the two translocases have clear differences in specificity. Secretion of the Tat-dependent proteins YwbN or PhoD is strictly dependent on the TatAyCy or TatAdCd translocases, respectively [57,69]. However, TatAdCd overexpression does permit translocation of YwbN, suggesting that this translocase has a more relaxed specificity than the TatAyCy translocase, which seems unable to translocate PhoD [70]. Both Tat systems recognize the same target determinants in signal peptides, but differ in the acceptance of particular proteins with RR-signal peptides as was shown when the translocases were heterologously expressed in *E. coli*.

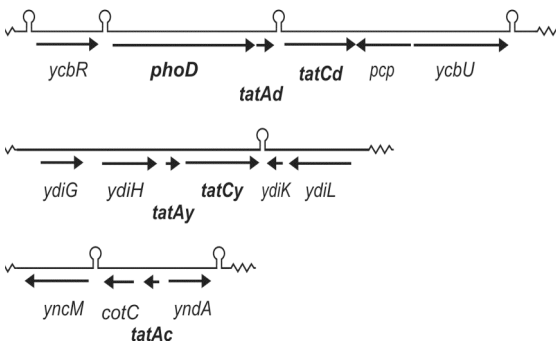

Figure 6. The *tat* genes present in *B. subtilis*, including the *phoD* gene upstream of *tatAd-tatCd* genes.

In this organism TatAyCy failed to transport TorA to the periplasm (**chapter 5**), whereas TatAdCd was able to facilitate TorA transport [58,71]. Possibly, this difference relates to differences in the complex sizes of the two translocases.

While TatAyCy forms complexes of ~200 kDa, TatAdCd forms slightly larger complexes of ~350 kDa, which may allow the formation of a larger channel.

TAT-DEPENDENTLY TRANSPORTED PROTEINS

Proteins destined for translocation contain so-called amino-terminal signal peptides. These signal peptides have a tripartite structure, consisting of an N-terminal part (n-region), a central hydrophobic part (h-region) and a C-terminal part (c-region). The c-region contains the recognition sequence for the signal peptidase that separates the signal peptide from the mature protein during or after translocation. When the principle of signal peptide function was first identified, it was expected that all identified signal peptides would direct proteins into the same Sec channel. However, especially the identification of the Tat pathway has added new flavour to signal peptide function as it showed that relatively small sequence motifs within signal peptides can serve to direct proteins into alternative pathways for protein transport. A distinctive feature of the Tat signal peptide is the presence of the twin-arginines that gave the name to the Tat pathway. The complete RR-motif in signal peptides was first defined as (S/T)-R-R-x-F-L-K [41,42,72]. However, it subsequently turned out that certain modifications to this motif are encountered in nature. These observations as well as the results from site-directed mutagenesis studies showed that the first arginine in the RR-motif can be replaced by lysine, whereas the second arginine appeared to be indispensable for productive protein transport via Tat [73]. The current consensus sequence for RR-motifs is R/K-R-X-#-#, where # indicates a hydrophobic residue and X can be any residue [74]. Furthermore, it was shown that the h-region of RR-signal peptides is generally less hydrophobic than the h-region of signal peptides that direct proteins into the Sec pathway, Figure 7 [75].

A third feature encountered in many RR-signal peptides is the presence of positively charged residues (arginine or lysine) in the c-region, which serve as a “Sec avoidance signal” to preclude mis-targeting of precursor proteins into the Sec pathway [75,76].

Computer-based prediction programs have been developed to identify potential RR-signal peptides (TatP, TatFind and TatPred; [77-79]). Nevertheless, prediction of Tat-dependent export is difficult, because the folded state of the transported protein is another important parameter in this process. Tat-dependently secreted proteins frequently need cofactors for activity or have the tendency to fold very rapidly, and

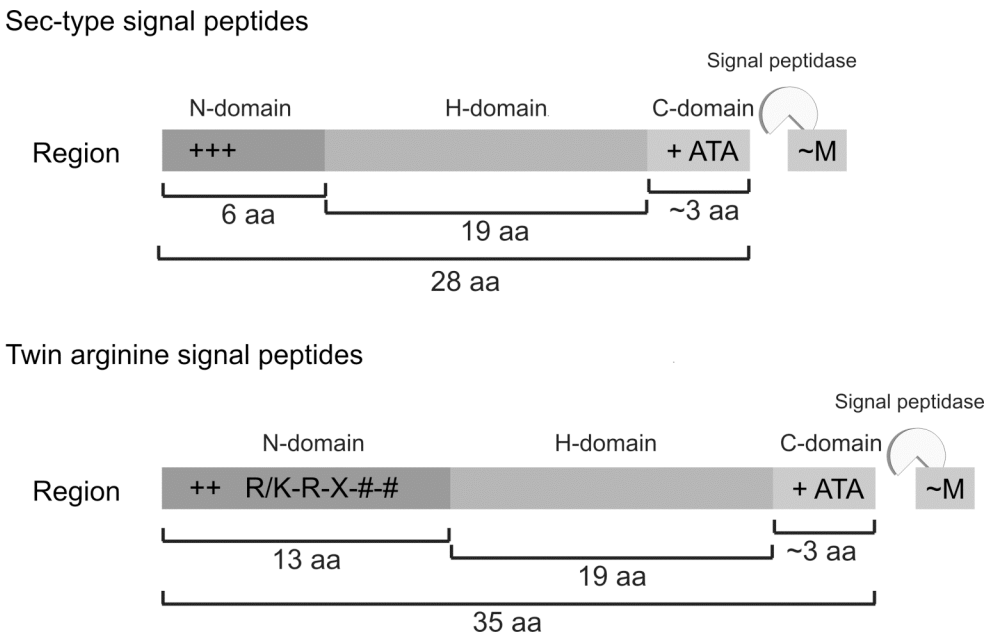

Figure 7. **N-terminal signal sequences**; (Top) Sec pathway directing signal peptide and (Bottom) Tat pathway directing signal peptide. ATA, , type I signal peptidase cleavage site; ~M, mature protein; aa, amino acids; +, indicates the charge of the region.

these folding features cannot be readily predicted [41,44]. Based on simple predictions of the presence of a signal peptide with a potential RR-motif, 69 proteins were identified that can potentially be exported via the Tat pathway of *B. subtilis* [19]. Nevertheless, it turned out difficult to identify strictly Tat-dependent secretory proteins. Proteomics analyses revealed a strict Tat-dependence for only one of the 69 predicted Tat-dependent proteins, while thirteen other proteins were shown to be exported Tat-independently. Subsequently, a few additional Tat-dependent secretory proteins were identified through specific analyses. This applied first to the Dyp-type peroxidase YwbN, which was provided with a C-terminal Myc-tag for identification [57]. The signal peptides of the *B. subtilis* QcrA and YkuE proteins were shown to confer Tat-dependent secretion of an agarase in *Streptomyces coelicolor*, which suggests that these proteins are also Tat-dependently secreted in *B. subtilis* [80]. The signal peptide of the esterase LipA displays a consensus RR-motif. Nevertheless, this protein was secreted Sec-dependently under standard laboratory growth conditions. Quite unexpectedly, a serendipitously identified LipA hyper-producing strain was shown to secrete this protein Tat-dependently via an overflow mechanism (**chapter 3**) [81]. Based on this latter result it was anticipated that more proteins might be identified when Tat-dependent protein secretion would be studied under the right conditions. Proof for this idea was found by growing cells in media of differing salinity. Thus, it was shown that the quality control

protease WprA was secreted Tat-dependently when cells were grown in medium with 6% salt, and that the cell wall hydrolase LytD was secreted Tat-dependently when cells were grown in medium lacking salt. (**chapter 4**). Together, these findings show that changes in the internal or external conditions of the cell may impact on Tat-dependent protein export. It will thus be a nice challenge for future research to identify conditions under which other secretory proteins with potential RR-signal peptides display a change from the Sec-dependent to the Tat-dependent secretion mode.

QUALITY CONTROL FACTORS AND CHAPERONES

Prior to transport, Tat-dependent proteins usually fold in the cytoplasm where they can bind co-factors or partner proteins [38,41,82,83]. To avoid the mis-targeting of these proteins or perhaps to avoid jamming of the Tat pathway, so-called quality control factors have evolved [84-86]. These quality control factors can arrest protein transport till precursor folding and assembly of co-factors have been completed. Alternatively, quality control factors can guide misfolded or immature proteins for disposal, Figure 8 [87,88]. In addition to saving energy and preventing a possible blockage or disruption of the Tat pathway, quality control factors may also be involved in the regulation of the transport process.

Quality control can be exerted already at early stages in transport via chaperones that, for example, facilitate co-factor insertion into transported precursor proteins. Similarly working related chaperones of this type are present in the cytoplasm of *E. coli*, namely TorD, DmsD, NapD and NarJ. Collectively, these chaperones have been termed redox enzyme maturation proteins, in short REMPs [89,90]. The best-studied quality control chaperone is the TorD protein of *E. coli*, which is dedicated to the maturation of TorA by facilitating co-factor binding [91-93]. For this purpose, a TorD dimer binds the signal peptide of TorD, thereby preventing TorA export from the cytoplasm. At the same time, TorD monitors the folding state of TorA. With bound TorD, the co-factor binding site of TorA is better accessible and the protein is protected from proteolysis [87,94]. Orthologues of the *E. coli* REMPs are present in many other species, including bacteria and archaea [89]. In *B. subtilis* REMP activity has not yet been demonstrated experimentally, but a homologue of NarJ is present in this organism, which may assist in the maturation of the nitrate reductase. Notably,

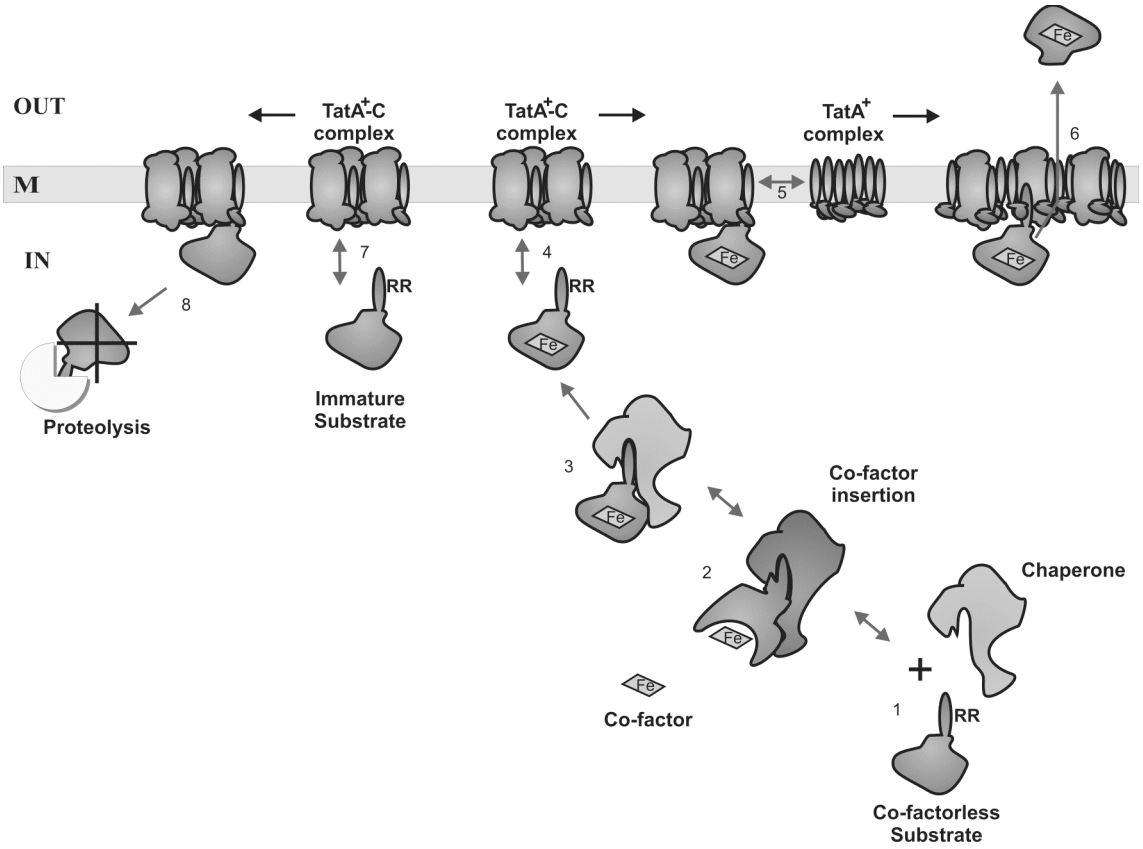

Figure 8. **Quality control factors and Chaperones**. Step 1, immature co-factorless protein is recognized and bound by a chaperone saving it from proteolysis; step 2-3, co-factor insertion and release; step 4-6, mature protein recognized by $TatA^{+}C$ receptor, $TatA^{+}$ pore formation and translocation; Step 7-8, interaction of Tat machine with immature protein leading to proteolysis.

however, the NarJ chaperone activity is not necessarily coupled to Tat-dependent transport, and the NarJ-dependent maturation of cytoplasmic nitrate reductase in *E. coli* occurs via a completely Tat-independent mechanism [89].

Another interesting example of quality control at an early stage of Tat-dependent protein export was derived from studies in which the role of general chaperones was assessed [95]. These studies revealed that the molecular chaperone DnaK is essential for the stability of a wide range of recombinantly produced endogenous and heterologous Tat substrates. Furthermore, the results showed that DnaK can be a limiting factor in Tat-dependent transport under conditions where the production level of the transported protein exceeds the capacity of the Tat translocase.

At late stages in Tat-dependent protein transport, quality control can be exerted via the Tat translocase complexes. First evidence for substrate proofreading prior to translocation was derived from experiments showing that the Tat-dependent export of co-factorless or unfolded proteins was retarded whereas folded proteins were effectively translocated [96,97]. In particular, the finding that the reduced

alkaline phosphatase PhoA (normally a Sec-dependent protein) fused to an RR-signal peptide was a poor substrate for Tat, while oxidized (folded) PhoA was effectively transported, led to the proposal that proofreading would occur at the level of the Tat translocase [97,98]. However, the actual role of Tat subunits in the proposed quality control in Tat-dependent export of oxidized PhoA was debated [99]. Clear evidence that the Tat pathway subunits proofread the folding state of substrate proteins, rejecting incorrectly or incompletely folded proteins was recently published by Matos *et al.* [88]. In this work, the fate of mutants of the *E. coli* Tat substrates NrfC and NapG was examined. Both are FeS proteins that bind their cofactors in the cytoplasm. Mutations in the FeS centres of NrfC and NapG blocked export, and resulted in rapid degradation. However, rapid degradation was prevented by *tat* mutations or when the RR-signal peptides were removed. This implies that the Tat machinery can proofread the folding state of NrfC and NapG and can trigger turnover of rejected molecules. Furthermore, degradation depended on the TatA or TatE subunits, suggesting that this process was initiated at a late stage in translocation after the initial binding of the signal peptide by TatBC.

Interestingly, also TatD turned out to be a critical factor for this quality control system as the NrfC and NapG variants lacking FeS centres were stabilised a *tatD* mutant [100]. This observation was remarkable since TatD was originally identified as a nuclease and discarded as a potential determinant for Tat translocation [54]. How exactly the *E. coli* Tat pathway is involved in quality control of substrate proteins remains to be determined. Also, it is presently not clear whether this mechanism is conserved in other organisms like *B. subtilis.* The only indication that this might be the case is that TatD is conserved in many bacteria.

POTENTIAL APPLICATION OF THE TAT PATHWAY FOR PROTEIN PRODUCTION

In recent years, it has been shown that the Tat pathway of *E. coli* has an excellent potential for commercial exploitation, since it can export a relatively wide range of folded proteins from the cytoplasm to the periplasm. This was first evidenced by export of the green fluorescent protein GFP via Tat [36,37]. Subsequent studies reported on the re-routing of folded, normally Sec-dependent proteins into the *E. coli* Tat pathway (*e.g.* PhoA and MBP; [101,102]), and the Tat-dependent export of engineered cytoplasmic proteins (*e.g.* a dimeric [2Fe-2S]-bridged form of TrxA [103]), heterologous enzymes [95,104] as well as a range of folded commercially interesting proteins (*e.g* single-chain Fv and heterodimeric F(AB) antibody fragments, and human tissue plasminogen activator [97,105]). These findings show that the Tat pathway of *E. coli* can tolerate a wide range of cargo proteins. However, due to the fact that *E. coli* is a Gram-negative bacterium, the proteins exported via Tat will generally end up in the periplasm, which complicates their large-scale downstream processing. This has focused interest on the exploitation of Tat pathways in Gram-positive bacteria, such as *Bacillus* end *Streptomyces* species [18,29,80,106]. Intriguingly, however, the usage of Tat pathways of these two species for the secretion of heterologous proteins has turned out to be difficult [107,108]. As to *B. subtilis*, despite the fact that this organism has a huge capacity for protein secretion, only one example of successful re-routing of a Sec-dependent protein into the Tat pathway has so far been described, while all other documented attempts were unsuccessful (**chapter 2,** [107]). Furthermore, no clear example of heterologous protein export via the Tat pathway of *B. subtilis* has been documented so far (**chapter 6**).

At present, it is not entirely clear why the *B. subtilis* Tat pathway is so restrictive as compared to the *E. coli* Tat pathway, but there are strong indications that this relates to a combination of a limited availability of Tat translocases [57], inefficient Sec-avoidance ([107], **chapters 2 and 6**), and possibly quality control leading to precursor degradation ([107], **chapters 2 and 6**). Importantly, the TatAdCd and TatAyCy translocases of *B. subtilis* seem to tolerate a fairly wide range of substrates when expressed in *E. coli* [58,71,109], which implies that these translocases should be able to do the same in *B. subtilis*. It thus seems that Sec avoidance and quality control are the key mechanisms that need to be better understood in order to unlock the *B. subtilis* Tat pathway for secretion of folded proteins of commercial value.

AIM AND SCOPE OF THIS THESIS – THE MINIMAL TAT TRANSLOCASE

The work described in this thesis is aimed at the functional analysis of "the minimal Tat translocase" as it is present in the Gram-positive bacterium *B. subtilis*. Specific aspects of the minimal Tat translocase that have been addressed are the possibilities and limitations for biotechnological exploitation, the biological function under different growth conditions, and structure-function relationships.

Chapter 1 provides an Introduction to the field of Tat-dependent protein translocation, pinpointing the different aspects that are relevant for this process. Proteins that are produced for commercial purposes in *B. subtilis* are commonly secreted via the Sec pathway. Despite its high secretion capacity, the secretion of heterologous proteins via the Sec pathway is often unsuccessful. Alternative secretion routes, like the Tat pathway, are therefore of interest.

In **Chapter 2**, the application potential of the two Tat pathways of *B. subtilis* was explored using commercially relevant or heterologous model proteins fused to RR-signal peptides from *B. subtilis*. Interestingly, the major extracellular serine protease of *B. subtilis*, subtilisin, was secreted via TatAyCy when provided with the signal peptide of the YwbN protein. All other attempts for protein re-routing to Tat remained however unsuccessful. The results suggest that cytoplasmic protein folding prior to translocation is probably a major determinant of Tat-dependent protein secretion in *B. subtilis*, as is the case in *E. coli*. However, the avoidance of the Sec pathway by engineered fusion proteins with RR-signal peptides seems frequently ineffective, resulting in their Tat-independent secretion.

Chapter 3 focuses attention on the previous finding that many predicted signal peptides of *B. subtilis* contain potential RR-motifs, which appear to be ignored or rejected by the Tat pathways of this organism [19]. A proteogenomics approach was employed to investigate the secretion mechanism of one of these proteins, the esterase LipA. For this purpose, a serendipitously obtained hyper-producing *B. subtilis* strain was used. The results show that, while LipA is secreted Sec-dependently under standard conditions, hyper-produced LipA is secreted predominantly Tat-dependently via a novel overflow mechanism. This overflow secretion mechanism focuses interest on the possibility that secretion pathway choice can be determined by environmental and intracellular conditions.

Chapter 4 builds on the observation that Tat seems to have evolved into a mainstream pathway for protein secretion in halophilic archaea [66]. This suggested that environmental salinity might also impact on Tat-dependent protein transport in bacteria

that are exposed to widely differing salt concentrations in their natural habitats. Since this does apply to the soil bacterium *B. subtilis*, the effects of environmental salinity on protein secretion by this organism were investigated. The results show that environmental salinity determines the specificity and usage of the *B. subtilis* Tat pathway. Thus, the WprA and LytD proteins were identified as novel Tat-dependently secreted proteins, which is consistent with the presence of RR-motifs in their signal peptides. Furthermore, high salinity induced a cooperative mode of action of the *B. subtilis* TatAdCd and TatAyCy translocases in the secretion of the Dyp-type peroxidase YwbN. Importantly, at low salinity, *tatAyCy* or *ywbN* mutants displayed significantly reduced exponential growth rates and severe cell lysis in the post-exponential growth stages, which revealed a critical role of Tat-dependently secreted YwbN in the acquisition of iron under these conditions. The findings described in chapter 4 show for the first time that environmental conditions, such as salinity, can determine the specificity, substrate spectrum and biological function of Tat-dependent protein secretion in a bacterium.

Chapter 5 describes functional studies on the two TatAC-type systems of *B. subtilis*. For this purpose, the TatAdCd and TatAyCy translocases were individually expressed in *E. coli*. The results show that they recognize similar signal peptide determinants. Both systems were shown to translocate GFP fused to three distinct *E. coli* Tat signal peptides, namely AmiA, DmsA and MdoD. One *E. coli* Tat substrate, TMAO reductase, was translocated by TatAdCd but not by TatAyCy. Over all, it seems that the TatAdCd and TatAyCy systems are not predisposed to recognize only specific Tat signal peptides, as originally suggested by their narrow substrate specificities in *B. subtilis*. Analysis of the Tat complexes revealed the presence of a TatAyCy complex together with a separate, homogeneous, ~200 kDa TatAy complex. The latter complex differs significantly from the corresponding *E. coli* TatA complexes, pointing to major structural differences between Tat complexes from Gram-negative and Gram-positive organisms.

Chapter 6 reports on the expression in *B. subtilis* of GFP fused to the Tat signal peptides of the *E. coli* AmiA, DmsA and MdoD proteins. Despite their effective translocation by heterologously expressed TatAdCd or TatAyCy complexes in *E. coli* (chapter 5), these GFP fusion proteins were not Tat-dependently secreted when expressed in *B. subtilis*. For some constructs Tat-independent secretion was demonstrated, which was particularly evident when *tat* mutant strains were grown under conditions of high salinity. The

results suggest that quality control mechanisms reject the GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to the Sec-dependent export of these fusion proteins. The Sec avoidance mechanism, but not the precursor rejection by Tat, is to some extentt overruled under conditions of high salinity.

Chapter 7 describes a mutational analysis of the TatAy subunit of the TatAyCy translocase. Several mutations in TatAy are shown to affect TatAy function or stability under standard laboratory growth conditions. Interestingly, most of these loss of function mutations did not interfere with the formation of TatAyCy complexes upon heterologous expression in *E. coli*. This is consistent with the finding that the mutant TatAy proteins show at least some activity under conditions of low salinity. Over all the results pinpoint residues that are critical for TatAy function and show that TatAyCy complexes are rather robust to mutations in TatAy.

All together, the studies in this thesis have increased our understanding of the mechanisms that control the usage, specificity and function of minimal TatAC translocases in *B. subtilis*. This is important not only because relatively little is known about the TatAC pathways, but also because such pathways could possibly be exploited for the production of high-value proteins especially in the biotechnological "workhorse" *B. subtilis*. The results provide novel leads to overcome the currently encountered bottlenecks in Tat-dependent protein secretion, specifically pointing at mechanisms for Sec avoidance and quality control as the major hurdles. One of the most striking findings described in this thesis is the conditional recognition of RR-signal peptides, for example under conditions of protein hyperproduction or when cells are grown in media of high or low salinity. Under iron-limited conditions, Tat function turned out to be growth rate-determining. This was even more so the case at low salinity where Tat function was also critical for cell survival in the post-exponential growth stage. This opens up the realistic possibility that the *B. subtilis* Tat pathway is far more important for survival in extreme ecological niches than it was thus far believed on the basis of growth under standardised laboratory growth conditions.

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Chapter 2

The twin-arginine signal peptide of *Bacillus subtilis* YwbN can direct either Tat- or Sec-dependent secretion of different 'cargo' proteins; secretion of active subtilisin via the *B. subtilis* Tat pathway

Marc Kolkman[#], René van der Ploeg[#], Michael Bertels, Maurits van Dijk, Joop van der Laan, Jan Maarten van Dijl and Eugenio A.B. Ferrari

[#]Both authors contributed equally to this work

ABSTRACT

Proteins that are produced for commercial purposes in *Bacillus subtilis* are commonly secreted via the Sec pathway. Despite its high secretion capacity, the secretion of heterologous proteins via the Sec pathway is often unsuccessful. Alternative secretion routes, like the Tat pathway, are therefore of interest. Two parallel Tat pathways with distinct specificities have previously been discovered in *B. subtilis.* To explore the application potential of these Tat pathways, several commercially relevant or heterologous model proteins were fused to the signal peptides of the known *B. subtilis* Tat substrates YwbN and PhoD. Remarkably, the YwbN signal peptide directed secretion of active subtilisin, a typical Sec substrate, via the *B. subtilis* TatAyCy route. By contrast, the same signal peptide directed Tat-independent secretion of the *Bacillus licheniformis* α-amylase (AmyL). Moreover, the YwbN signal peptide directed secretion of SufI, an *Escherichia coli* Tat substrate, in a Tat-independent manner, most likely via Sec. Our results suggest that cytoplasmic protein folding prior to translocation is probably a major determinant of Tat-dependent protein secretion in *B. subtilis*, as is the case in *E. coli*. We conclude that future applications for the Tat system of *B. subtilis* will, most likely, involve commercially interesting proteins that are Sec-incompatible.

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INTRODUCTION

Bacillus subtilis is an industrially important bacterium that is commonly used as a host for the commercial production of proteins, especially enzymes such as proteases, amylases and lipases [1-4]. One of the major advantages of *B. subtilis* over many other bacteria is its ability to secrete high amounts of proteins into the growth medium, allowing efficient recovery of these protein products. Unfortunately, the secretion of heterologous proteins is often inefficient or not successful at all, and this applies in particular to proteins from Gram-negative bacteria or eukaryotes [5]. Various potential bottlenecks in the secretion of heterologous proteins have been identified. Many of the encountered problems relate to the particular properties of the secreted protein, the secretion pathway, or both [6,7]. Notably, in most documented cases the general secretion (Sec) pathway was used, which is known for its high capacity to transport proteins from the cytoplasm to the growth medium. Importantly however, *B. subtilis* contains several alternative routes for protein export and secretion, one of which is known as the twin-arginine translocation (Tat) pathway [8-11]. This pathway may offer an alternative secretion route for Sec-incompatible proteins in bacilli.

The Tat pathway is present in many bacteria and chloroplasts [12-17]. It is distinguishable from the Sec pathway by two major features. Firstly, preproteins are directed to the Tat pathway by signal peptides that bear a characteristic sequence motif: the twin-arginine (RR) motif that is essential for signal peptide recognition by the Tat translocation machinery [18-20]. Secondly, proteins can be exported via Tat in a folded state, whereas the Sec pathway exclusively transports proteins in an unfolded state [21-25]. To date, active Tat pathways have been identified in many Gram-negative and Gram-positive bacteria. Interestingly, these two classes of bacteria seem to contain Tat translocases of similar, but non-identical subunit composition. Minimal Tat translocases have been identified in Gram-positive bacteria, such as *B. subtilis*. These translocases consist of two subunits named TatA and TatC [11,26]. By contrast, the Tat translocases from Gram-negative bacteria, mycobacteria and streptomycetes contain an additional third subunit, named TatB. To date, the *Escherichia coli* TatABC system is best characterized. The TatA, TatB and TatC subunits are indispensable for translocase activity [19,27-29]. Notably, a fourth Tat subunit from *E. coli*, TatE, is a paralogue of TatA and this subunit can complement for the absence of TatA [30]. In minimal TatAC translocases, the TatA protein is thought to be bifunctional [31], performing the functions of both the TatA and TatB

proteins in the TatABC translocases. Notably, two minimal TatAC systems with distinct substrate specificities have been reported for *B. subtilis* [11,26,32]. These systems have been termed TatAdCd and TatAyCy. The view that the *B. subtilis* TatA subunits are bifunctional is supported by the recent observation that the TatAd subunit can complement for the absence of the *E. coli* TatA or TatB proteins [33].

The genes encoding the two *B. subtilis* TatAC systems are located at distinct genomic positions [10]. The *tatCd* and *tatCy* genes are preceded by the cognate *tatAd* and *tatAy* genes, respectively. A third *tatA* gene, denoted *tatAc*, is not genetically linked to another *tat* gene and no function has been identified for the TatAc protein. The TatAyCy translocase is essential for secretion of YwbN, a protein belonging to the family of heme-containing DyP-type peroxidases [11]. By contrast, the TatAdCd translocase is essential for secretion of the PhoD phosphodiesterase, which is produced exclusively upon phosphate starvation [10,11]. The *phoD* gene is located upstream of the *tatAdCd* genes, whereas the *ywbN* gene is not genetically linked to the *tatAyCy* genes.

A search for RR-signal peptides encoded by the genome of *B. subtilis* revealed several proteins that could potentially use the Tat pathway for secretion [8,34]. However, until now, strictly Tat-dependent secretion in *B. subtilis* has only been demonstrated for PhoD and YwbN. Recently, it was shown that two additional RR-signal peptides, which were derived from the *B. subtilis* QcrA and YkuE proteins, can direct Tat-dependent secretion of agarase in *Streptomyces lividans*. This indicates that QcrA and YkuE are also transported by the *B. subtilis* Tat machinery [35]. So far, there is little published data available concerning the secretion of heterologous proteins via the Tat pathway of *B. subtilis*. The *E. coli* phytase AppA was shown to be exported TatAdCd-dependently when fused to the RR-signal peptide of PhoD [36], and also Tat-dependent secretion of the Green Fluorescent Protein (GFP) was reported, albeit in an inactive form [22]. Furthermore, when expressed in *E. coli*, the TatAdCd translocase was able to translocate the *E. coli* Trimethylamine N-oxide reductase (TorA), or GFP fused to the DmsA or TorA RR-signal peptides [33,37]. These findings indicate that the *B. subtilis* Tat machinery is capable of translocating heterologous and tightly folded proteins. In the present studies, we have further explored the capabilities for transport of homologous and heterologous proteins via the Tat pathways of *B. subtilis*. For this purpose, fusions were made between reporter proteins that are normally secreted via the Sec system of *Bacillus* species or the Tat system of *E. coli*, and the RR-signal peptides of the *B. subtilis* Tat substrates PhoD or YwbN.

RESULTS

Tat-dependent secretion of subtilisin directed by the YwbN signal peptide.

The Tat pathway stands out by its ability to transport folded proteins across membranes, while the Sec pathway transports proteins in an unfolded state. Nevertheless, a few examples of re-routing of normally Sec-dependently secreted proteins into the Tat pathway have been described for *E. coli* [22,38-40]. These include the maltose-binding protein MalE and the alkaline phosphatase PhoA. However, Tat-dependent transport of these proteins to the periplasm requires their folding prior to translocation. To investigate whether a commercially highly relevant Sec-dependent protein can be re-routed into the Tat pathway of *B. subtilis*, we used the *B. subtilis* subtilisin, encoded by the *aprE* gene, as a reporter. To direct subtilisin towards the *B. subtilis* Tat machinery, this protein was fused to the genuine RR-signal peptides of *B. subtilis* PhoD or YwbN. The respective hybrid genes were integrated into the chromosomal *aprE* promoter regions of Tat proficient (Tat^+) or completely Tat deficient (ΔTat) *B. subtilis* strains. Both strains contained the *degU32*(hy) mutation for high-level expression of the fusion proteins, and lacked five major proteases (AprE, NprE, Epr, IspA, Bpr) allowing the detection of secreted subtilisin on skim milk plates. As shown in Figure 1A, fusion of the YwbN signal peptide (SP_{YwbN}) to subtilisin resulted in complete Tat-dependent secretion of subtilisin: no zone of subtilisin activity was detectable around colonies of the Tat deficient strain producing the SP_{YwbN}–subtilisin fusion protein, while a clear zone of subtilisin activity was detectable around colonies of the Tat proficient strain producing this fusion protein. A completely different result was obtained when subtilisin was fused to the PhoD signal peptide (SP_{PhoD}). In this case, active subtilisin was secreted by both the Tat proficient strain and the ΔTat strain. Thus, the PhoD signal peptide directed the Tat-independent export of subtilisin, as was also observed for subtilisin secretion directed by its authentic signal peptide (SP_{AprE}; Fig. 1A). As expected, no active subtilisin was detected in the extracellular environment of cells that produced this protein without a signal peptide (Fig. 1A).

Secretion of subtilisin with the YwbN signal peptide is TatAyCy-dependent.

It was previously shown that the TatAyCy translocase is specifically required for the secretion of YwbN [11]. To examine whether the subtilisin secretion directed by the YwbN signal peptide would also be TatAyCy-dependent, we expressed the SP_{YwbN}-subtilisin fusion protein in *tatAyCy* or *tatAdCd* mutant strains . Secretion of

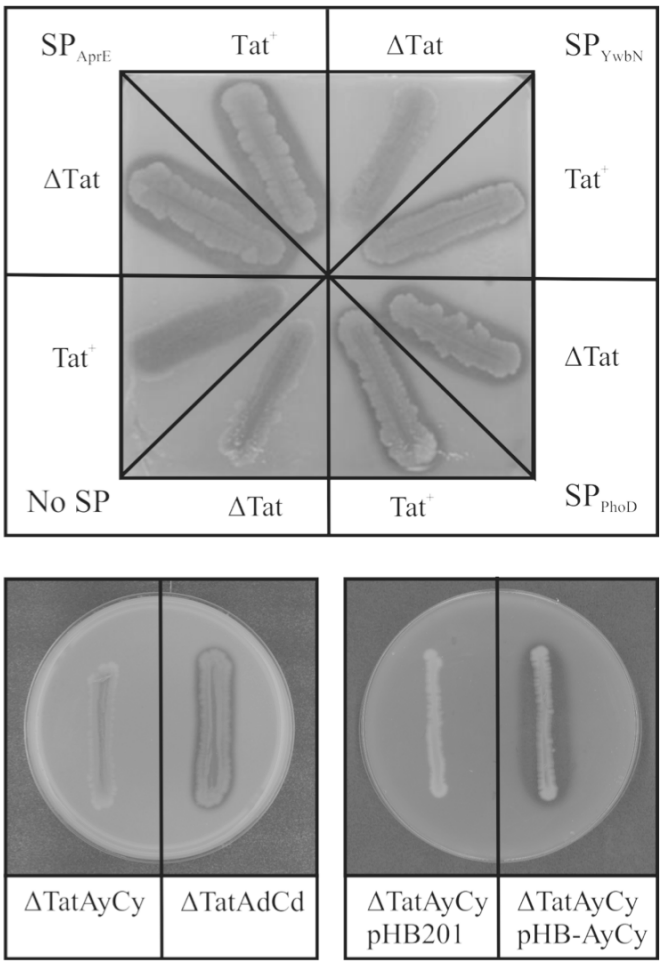


Figure 1. Tat-dependent secretion of subtilisin. (A, upper panel and B, the two panels below)

(A) The secretion of active subtilisin by Tat proficient (Tat^+) or Tat deficient (ΔTat) derivatives of *B. subtilis aprE*, *nprE*, *epr*, *ispA*, *bpr*, *amyE* was monitored by halo formation on skim milk plates**.** Pro-subtilisin was fused to the PhoD signal peptide (SP_{PhoD}), the YwbN signal peptide (SP_{YwbN}) or the signal peptide of subtilisin (SP_{AprE}). Alternatively, pro-subtilisin was produced without a signal peptide (No SP).

(B) Secretion of active subtilisin directed by SP_{YwbN} was monitored in *B. subtilis tatAyCy* (ΔTatAyCy), *B. subtilis tatAdCd* (ΔTatAdCd) shown in the left panel, and *B subtilis tatAyCy* with the empty vector pHB201 (ΔTatAyCy pHB201), or the complemented strain *B. subtilis tatAyCy* containing pHB-AyCy (ΔTatAyCy pHB-AyCy) shown in the panel on the right, as described for panel A

active subtilisin was again assayed by halo formation on skim milk plates (Fig. 1B). The results showed that the *tatAdCd* mutant was still capable of subtilisin secretion, as clear halos were formed around colonies producing SP_{YwbN}-subtilisin. In contrast, no halos were formed around colonies of the *tatAyCy* mutant expressing the SP_{YwbN}-subtilisin fusion protein. This indicated that subtilisin secretion directed by the YwbN signal peptide is TatAyCy dependent. To confirm this idea, we investigated whether subtilisin secretion directed by the YwbN signal peptide in a *tatAyCy* mutant could be restored by introduction of a plasmid expressing the *tatAyCy* genes. This was indeed the case (Fig. 1B), showing that the YwbN signal peptide directs subtilisin secretion in a TatAyCy dependent manner.

Tat-independent secretion of the *Bacillus licheniformis* α-amylase AmyL directed by the YwbN signal peptide.

To investigate whether the signal peptide of YwbN would also direct the secretion of Sec-dependent proteins other than subtilisin, we fused this signal peptide to the mature part of the *Bacillus licheniformis*

α-amylase (AmyL). Next, secretion of AmyL directed by the YwbN signal peptide was tested in *B. subtilis* strains lacking either *tatAyCy* or *tatAdCd*. The secretion of active amylase by these *tat* mutant strains or the Tat proficient control strain was monitored by growth on starch-containing plates and subsequent staining of the plates with iodine (Fig. 2). Unexpectedly, both *tat* mutant strains and the wild-type control showed similar levels of YwbN-directed secretion of the AmyL amylase. Furthermore, expression of SP_{YwbN}-AmyL in a strain lacking both the TatAdCd and the TatAyCy translocases resulted in the secretion of active α-amylase at a similar level as observed for the Tat proficient control strain (data not shown). This shows that the YwbN signal peptide does not direct Tat-dependent AmyL secretion in *B. subtilis*.

Tat-independent secretion of *E. coli* SufI with the YwbN signal peptide.

The results reported above show that the YwbN signal peptide was capable of directing both Tat-dependent and Tat-independent export of different reporter proteins that are normally secreted via the Sec pathway. This raised the question whether the YwbN signal peptide would be capable of directing a heterologous Tat-dependent reporter protein into the *B. subtilis* Tat pathway. To answer this question, we employed the SufI protein from *E. coli*, which is targeted to the periplasm via the Tat pathway [41]. SufI was fused to the YwbN signal peptide and

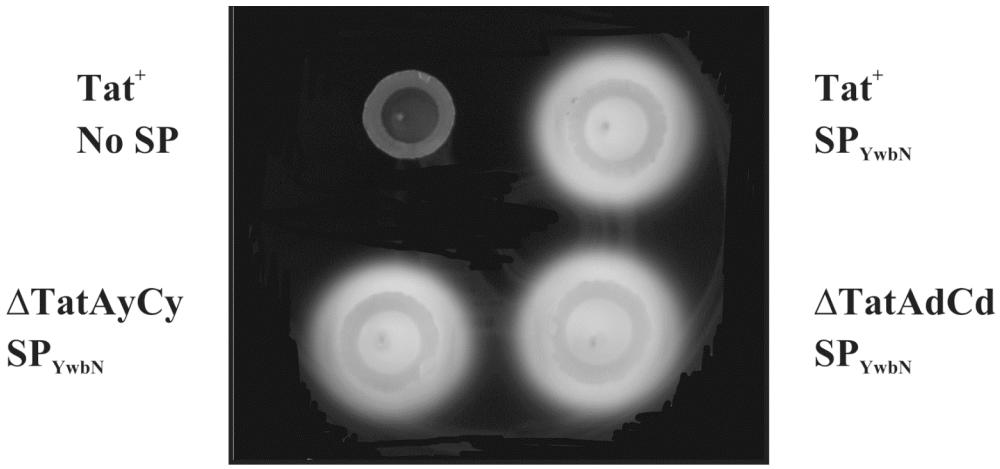


Figure 2. Tat-independent secretion of *B. licheniformis* α-amylase with the signal peptide of YwbN. The secretion of active AmyL by Tat proficient (Tat^+), TatAdCd deficient (ΔTatAdCd) or TatAyCy deficient (ΔTatAyCy) derivatives of *B. subtilis aprE*, *nprE*, *epr*, *ispA*, *bpr*, *amyE* was visualized by halo formation on an HI-plate containing 0.2% starch. AmyL was fused to the YwbN signal peptide (SP_{YwbN}). Alternatively, AmyL was produced without a signal peptide (No SP). After 24 hours of growth at 37°C, the plates were stained with iodine.

the secretion of SufI in *B. subtilis* Tat deficient or Tat proficient strains was analyzed by SDS-PAGE and Western blotting using cellular and growth medium fractions. As shown in Figure 3, the YwbN signal peptide directed Tat-independent secretion of SufI in *B. subtilis*. Furthermore, a significant amount of the SufI produced remained cell-associated. These findings suggested that the YwbN signal peptide was capable of directing at least some SufI secretion via the Sec pathway of *B. subtilis*, despite the fact that this protein is a Sec incompatible Tat substrate in *E. coli.* To investigate whether SufI secretion in *B. subtilis* could be directed with a Sec-specific signal peptide, a hybrid SufI precursor was constructed using the signal peptide of subtilisin (SP_{AprE}). This signal peptide also directed SufI secretion in a Tat-independent manner, but much more effectively than the YwbN signal peptide (Fig. 3). This was evident from the ratio of cellular over secreted SufI, which was much higher for the strain expressing the SP_{YwbN}-SufI hybrid precursor than for cells expressing the SP_{AprE}-SufI precursor. Furthermore, the SP_{YwbN}-SufI precursor was detectable in the cellular fraction, while the SP_{AprE}-SufI precursor could not be detected. Nevertheless, mature SufI was clearly detectable in the cellular fraction of the SP_{AprE}-SufI producing Tat proficient and Tat deficient strains. Finally, when SufI

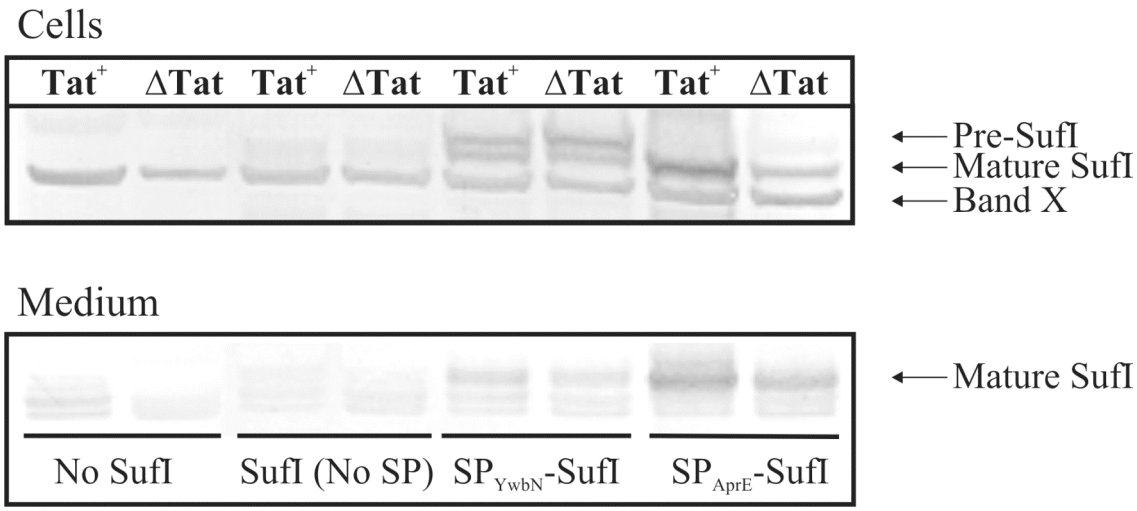


Figure 3. Tat-independent secretion of *E. coli* SufI. The secretion of SufI by Tat proficient (Tat^+) or Tat deficient (ΔTat) derivatives of *B. subtilis aprE*, *nprE*, *epr*, *ispA*, *bpr*, *amyE* was analysed by Western blotting. SufI was either not expressed (No SufI), expressed without a signal peptide (SufI (No SP)), with the signal peptide of YwbN (SP_{YwbN}-SufI), or with the signal peptide of subtilisin (SP_{AprE}-SufI) as indicated. Samples were taken from cultures in the stationary phase. After centrifugation, proteins in the cellular and growth medium fractions were separated by SDS-PAGE. Western blotting and immuno-detection were performed with specific anti-SufI antibodies. An unidentified cross-reacting band is marked as band X.

was expressed without a signal peptide barely any SufI protein was detectable, suggesting that it was not efficiently synthesized without a signal peptide, or rapidly degraded.

DISCUSSION

The *B. subtilis* Sec system has a massive capacity for exporting a wide range of proteins into the extracellular milieu [4,5]. This pathway is particularly effective in secreting homologous proteins and proteins from closely related Gram-positive species, like other bacilli. Notably, proteins transported via the Sec pathway pass the membrane translocation channel in an unfolded state, which makes these proteins vulnerable to post-translocational degradation [8,9,42]. Indeed, post-translocational folding defects set important limits to the yields of heterologous proteins secreted via the Sec pathway of *B. subtilis* [5,43-45]. This prompted us to investigate the capabilities of the Tat pathway using *Bacillus* enzymes of major commercial significance as reporter proteins. Post-translocational folding of AmyL from *B. licheniformis* in *B. subtilis* is known to be relatively inefficient and, consequently, significant amounts of translocated protein are lost due to proteolysis [46,47]. In such a case, folding in the cytoplasm and translocation via the Tat pathway might be an interesting alternative route for secretion. Our present results show that subtilisin can indeed be re-targeted from the Sec to the Tat pathway, but this requires the twin-arginine signal peptide of YwbN. Consistent with the function of this signal peptide in the translocation of YwbN via the TatAyCy translocase, subtilisin was secreted via this particular Tat translocase of *B. subtilis* when provided with the YwbN signal peptide. The TatAdCd translocase appeared not to be involved in this process. Surprisingly, the fusion of subtilisin to the twin-arginine signal peptide of PhoD did not result in secretion via Tat. Nevertheless, subtilisin was secreted with the aid of SP_{PhoD}, most likely via the Sec pathway. This suggests that SP_{PhoD} does not include an effective Sec avoidance signal. Such a Sec avoidance signal function was previously shown for positively charged residues at the interface of the h- and c-regions of twin-arginine signal peptides [40,48-50]. It thus seems that the Lys residue located fourteen residues N-terminally of the signal peptidase I recognition sequence of SP_{PhoD} in our constructs does not efficiently serve this role. Although no exclusive TatAdCd-dependent secretion of subtilisin could be observed when this protein was fused to the PhoD signal peptide, we cannot rule out the

possibility that some subtilisin was secreted via TatAdCd in parallel to secretion via Sec. These observations have interesting implications regarding subtilisin folding. The generally accepted view is that the Tat pathway translocates mainly folded proteins [51,52], but some exceptions to this general rule appear to exist as evidenced from the translocation of unfolded DHFR via the thylakoidal Tat system [25] and the Tat-dependent translocation of unstructured, small, hydrophilic proteins in *E. coli* [53]. Folding of subtilisin is known to be coordinated by the pro-peptide of this protein, which acts as a folding catalyst. Thus, it is conceivable that pro-subtilisin folds in the cytoplasm prior to translocation via TatAyCy if this protein is provided with the YwbN signal peptide. If so, SP_{YwbN} might increase the folding kinetics of subtilisin. However, it is difficult to assess whether pre-translocational folding of SP_{YwbN}–subtilisin did indeed occur, or whether this precursor was translocated via TatAyCy in an unfolded state. It is not clear what happens in the case of the SP_{PhoD}-subtilisin precursor, but three alternative explanations are conceivable. Firstly, the TatAdCd translocase is predominantly synthesized under conditions of phosphate starvation [10] although some activity is detectable in rich media [22]. Thus, the availability of TatAdCd may have been limiting under the conditions tested, which might have favored translocation via Sec. Secondly, the SP_{PhoD}-subtilisin precursor may not be able to fold in the cytoplasm resulting in rejection by TatAdCd, but allowing acceptance by Sec. This would, for example, be the case if SP_{PhoD} can not effectively increase the folding kinetics of subtilisin to allow Tat-dependent transport. Thirdly, the TatAdCd translocase may be less capable of handling proteins that do not fold effectively in the cytoplasm. Notably, these possibilities are not mutually exclusive, which makes it hard to pinpoint the reason for the observed differences. Clearly, cytoplasmic folding of proteins re-routed from Sec to Tat is a strict requirement in *E. coli*, as was shown for GFP [54-56], the *E. coli* alkaline phosphatase PhoA [39] and the *E. coli* maltose binding protein Mbp [38,40]. It seems therefore likely that the same will apply in *B. subtilis*. If this view is correct, the observed Tat-incompatibility of AmyL will probably relate to an inability of this protein to fold in the cytoplasm. An important difference here is that AmyL requires extracytoplasmic folding catalysts, like the lipoprotein PrsA [57], whereas subtilisin has its own intramolecular folding catalyst, the pro-peptide [58]. It has been proposed that the *E. coli* SufI protein requires translocation via Tat, not because it is a co-factor-containing protein, but rather because it folds too rapidly in the cytoplasm [13,41]. This raised the expectation that it would be fairly easy to translocate SufI via

the Tat pathway of *B. subtilis* if this protein were provided with an appropriate *Bacillus* twin-arginine signal peptide. It was anticipated especially that the YwbN signal peptide might perform well for SufI translocation, because this signal peptide directs strictly Tat-dependent secretion of subtilisin. Much to our surprise however, only Tat-independent secretion of SufI was observed in *B. subtilis*, and substantial amounts of the SP_{YwbN}-SufI precursor remained detectable in the cell. This protein was in fact even far better secreted when fused to the subtilisin signal peptide (SP_{AprE}), which is a genuine Sec signal peptide. These findings suggest that SufI does not fold rapidly in the milieu of the *B. subtilis* cytoplasm. If so, this could be due to (i) absence of an as yet unidentified folding catalyst, (ii) inhibition of SufI folding by an unidentified cytoplasmic factor, or (iii) slow cytoplasmic folding of SufI caused by the AprE and YwbN signal peptides. The observed Tat-independent secretion of SufI also raises the question whether "Tat proofreading" occurs in *B. subtilis*, as reported for Tat-dependent protein transport in *E. coli* [59-61]. This would require dedicated chaperones such as DmsD or TorD of *E. coli* [62-64]. Furthermore, the Tat-independent secretion of SufI directed by SP_{YwbN} suggests that this twin-arginine signal peptide of *B. subtilis*, like SP_{PhoD}, does not contain an effective Sec-avoidance signal. The latter view is supported by the observed Tat-independent secretion of AmyL fused to SP_{YwbN}, and by the fact that this signal peptide completely lacks positively charged residues between the h- and c-regions.

Taken together, our studies with subtilisin show that a commercially important Sec-dependent protein can be re-routed into the Tat pathway of *B. subtilis,* if it is provided with the right twin-arginine signal peptide. Nevertheless, the capability of the Tat pathway of *B. subtilis* to transport heterologous or re-routed "cargo" proteins, or even heterologous Tat-dependent proteins like SufI, seems to be fairly limited. For example, we tested GFP, TEV protease, BBI, *E. coli* AppA, and human trypsin without observing efficient or strictly Tat-dependent secretion (data not shown). On the other hand, inactive GFP is secreted via Tat of *B. subtilis*, and secretion of the *E. coli* phytase AppA seems possible with the signal peptide of PhoD [36]. Our results suggest that cytoplasmic folding is probably a major issue for Tat-dependent protein secretion in *B. subtilis*, as is the case in *E. coli*. Ironically, re-routing from Sec to Tat also "re-routes" the folding problem from the post-translocational stage to the pre-translocational stage. However, this may turn out to be an advantage, because it is probably easier to express appropriate heterologous folding catalysts in the cytoplasm than at the extracytoplasmic

membrane surface of *B. subtilis*. Thus, despite the various examples of Tat-incompatibility reported in our present manuscript, we do foresee possible interesting applications for the Tat system of *B. subtilis*, for example, in the production of commercially relevant Sec-incompatible proteins.

MATERIALS AND METHODS

Plasmids, bacterial strains and media.

Two *E. coli* strains were used: *E. coli* DH5α (*sup*E44 Δ*lac*U169 (Φ80*lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1), and *E. coli* TOP10 (F^- mcrA Δ*(mrr-hsd*RMS-*mcr*BC*)* Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R araD139, Δ(*ara-leu*)7697 *gal*U *gal*K *rpsL* (Str^R) *end*A1 *nup*G; Invitrogen). All *B. subtilis* strains that were used are derivatives of the sequenced *B. subtilis* 168 strain [65]. Plasmids used in this study are listed in Table I. *E. coli* strains were grown on 2xTY medium (Bacto tryptone, 16 g/l; yeast extract, 10 g/l; and NaCl, 5 g/l). *B. subtilis* strains were grown in TSB (Tryptone Soya Broth from Oxoid, 30 g/l), or on HI-agar (Heart Infusion agar from Difco, 40 g/l). When appropriate, media were supplemented with skim milk (1.5%), starch (0.2%), spectinomycin (100 µg/ml), kanamycin (20 µg/ml), erythromycin (2 µg/ml *B. subtilis*; 100 µg/ml *E. coli*), chloramphenicol (5 µg/ml) and/or tetracycline (10 µg/ml). Starch was stained with Lugol's iodine solution (1 g crystalline iodine, 2 g KI, 300 ml H_2O).

DNA techniques.

Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen) according to instructions of the supplier. Procedures for DNA restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook *et al.* [66]. Restriction enzymes and T4 DNA ligase were purchased from Invitrogen. PCR was carried out with High Fidelity Platinum Taq polymerase (Invitrogen). Transformation of competent *B. subtilis* was performed as previously described [67]. Protease deficient *B. subtilis* Δtat, Δ*tatAdCd* or Δ*tatAyCy* strains were obtained by transformation of protease mutant strains with linearized pRACd2 and/or pRACy3 [11,34]. Transformants were selected for spectinomycin and/or kanamycin resistance, and *tat* mutations were verified by PCR.

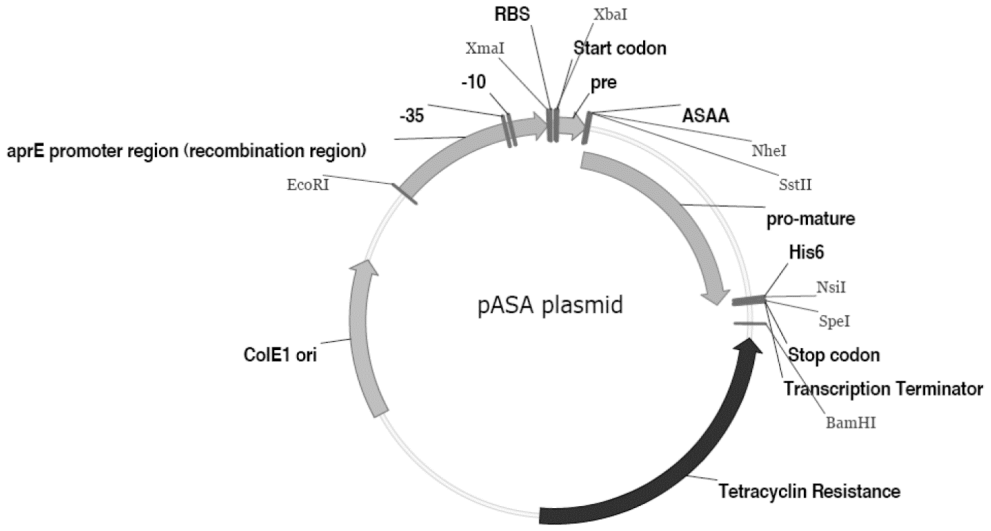
SDS-PAGE and Western blot analysis.

SDS-PAGE was carried out as described by Laemmli [68]. After separation by SDS-PAGE, proteins were transferred to a PVDF membrane (Roche Diagnostics). Immunoblotting was performed with antibodies against SufI (kindly provided by

Tracy Palmer). Bound antibodies were detected with alkaline phosphatase-labeled conjugate and the BM Chromogenic Western Blotting kit (Roche Diagnostics) according to the instructions of the manufacturer.

Construction of the pASA plasmids.

Plasmid pASA and its derivatives (Table I) were constructed by ligation of various DNA fragments obtained through PCR. Part of the plasmid pCR2.1-TOPO (Invitrogen), including the ColE1 origin, was used as a basis for pASA construction. The tetracycline resistance cassette in pASA was amplified from pDG1515 [69]. The *aprE* promoter region and *aprE* gene were amplified from *B. subtilis* 168 chromosomal DNA. In the resulting pASA plasmid (Fig. 4), signal sequences can be cloned directly downstream of the *aprE* ribosome binding site (RBS) as *Xba*I - *Nhe*I fragments, without affecting the integrity of this RBS. Genes for secretory reporter proteins can be cloned using the restriction sites *Sst*II-*Spe*I or *Sst*II-*Nsi*I. By using the *Nsi*I site, it is possible to fuse the cloned




```
  XmaI       XbaI          NheI     SstII           NsiI         SpeI          BamHI
.CCCGGG<RBS>TCTAGAATG<pre>GCT AGC GCC GCG GNN<pro-mat.>ATGCAT<His4>CACTAGTNN<Term.>GATCC.
             Start  Ala Ser Ala Ala            MettHIS     HIS Stop
```

Figure 4. Schematic representation of plasmid pASA. pASA contains the ColE1 ori for replication in *E. coli*, a tetracycline resistance gene for selection in *E. coli* and *B. subtilis*, a 500 bp fragment from the *B. subtilis* chromosome containing the *aprE* promoter in front of the cloning sites for signal peptide sequences (pre) and target genes (pro-mature), and the transcription terminator of the *aprE* gene. Restriction sites for cloning are shown in the plasmid map, and the fusion sites for cloned signal sequences and target genes are shown underneath the plasmid map.

secretory gene in-frame to a (C-terminal) His-tag sequence, making it possible to detect and/or purify the target protein. The amino acid sequence ASAA (Ala – Ser – Ala – Ala) is present as the standard signal peptidase I cleavage site, linking the signal peptide and the reporter protein. Finally, pASA contains the *aprE* transcription terminator downstream of the target genes.

The signal sequences of *ywbN* (SP_{YwbN}), *aprE* (SP_{AprE}) and *phoD* (SP_{PhoD}) were amplified from the chromosome of *B. subtilis* 168 and fused to the *aprE* gene, resulting in the plasmids pASA4, pASA5, pASA6, respectively. Accordingly, pASA4 encoded the YwbN signal peptide: MSDEQKKPEQIHRRDILKWGAMAGA AVAIGASGLGGLAPLV **ASA/A**; pASA5 encoded the AprE signal peptide: MRSKKLWISLLFALTLIFTMAFSNMS**A SA/A**; and pASA6 encoded the PhoD signal peptide: MAYDSRFDEWVQKLK EESFQNNTFDRRKFIQGAGKIAGLSLG LTIAQS**ASA/A** (the signal peptidase I recognition sequence ASA and the +1 Ala residue of the mature protein are indicated in bold). Furthermore, pASA3 was constructed as a negative control containing the *aprE* gene without a signal sequence. Next, the *aprE* gene in pASA4 was replaced with the *amyL* gene from *Bacillus licheniformis*, or the *sufI* gene from *E. coli* K12. This resulted in plasmids pASA4AmyL and pASA4SufI, respectively. Furthermore, the copies of the *aprE* gene in pASA3 and pASA5 were replaced with the *sufI* gene, respectively resulting in plasmids pASA3SufI and pASA5SufI. All pASA plasmids were obtained upon transformation of *E. coli*

Table I. Plasmids

Plasmids	Properties	Reference
pASA	*B. subtilis* integration plasmid; replicates in *E. coli* (ColE1 ori); Tc^r ; Fig. 4	This study
pASA3	pASA plasmid encoding proAprE without a signal peptide	This study
pASA4	pASA plasmid encoding SP_{YwbN}-proAprE	This study
pASA5	pASA plasmid encoding SP_{AprE}-proAprE	This study
pASA6	pASA plasmid encoding SP_{PhoD}-proAprE	This study
pASA4AmyL	pASA plasmid encoding SP_{YwbN}-AmyL	This study
pASA3SufI	pASA plasmid encoding E. coli SufI without a signal peptide	This study
pASA4SufI	pASA plasmid encoding SP_{YwbN}-SufI	This study
pASA5SufI	pASA plasmid encoding SP_{AprE}-SufI	This study
pRACd2	pUC21 derivative for the replacement of *tatAd-tatCd*; 5.7 kb; Ap^r; Km^r	Jongbloed *et al.*, 2002
pRACy3	pUC21 derivative for the replacement of *tatAy-tatCy*; 5.8 kb; Ap^r; Sp^r	Jongbloed *et al.*, 2002
pHB201	Shuttle vector for *E. coli* and *B. subtilis* with a p59 promoter; Em^r and Cm^r	Bron *et al.*, 1998
pHB-AyCy	pHB201 plasmid encoding the *tatAy-tatCy* operon	This study

TOP10 and selection for tetracycline resistance. The correct construction and absence of PCR mistakes were verified by sequencing (ServiceXS, Leiden, the Netherlands). Next, competent *B. subtilis* cells (*aprE*, *nprE*, *epr*, *ispA*, *bpr*, *amyE*) were transformed with the different pASA plasmids. Since the pASA plasmids lack replication functions for *B. subtilis*, all tetracycline-resistant transformants contained a pASA copy that was integrated into the chromosomal *aprE* promoter region by a single cross-over recombination event. To induce expression of the signal sequence-reporter gene fusions on the chromosomally integrated pASA plasmids, the *degU32*(hy) mutation was introduced into the different strains by transformation with DNA from *B. subtilis* MD300 and subsequent selection for tetracycline and kanamycin resistant transformants [70].

Construction of the pHB-AyCy plasmid.
To construct plasmid pHB-AyCy, the *B. subtilis* 168 *tatAy-tatCy* genes were amplified by PCR as described by Jongbloed *et al.* 2004. Next, the amplified fragment was cloned in the *Sma*I and *EcoR*I sites of pHB201 [71], resulting in pHB-AyCy. Correct amplification of the *tatAy* and *tatCy* genes was verified by sequencing.

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Chapter 3

Overflow of a hyper-produced secretory protein from the *Bacillus* Sec pathway into the Tat pathway for protein secretion as revealed by proteogenomics

Thijs R.H.M. Kouwen, René van der Ploeg, Haike Antelmann, Michael Hecker, Georg Homuth, Ulrike Mäder, Jan Maarten van Dijl

ABSTRACT

Bacteria secrete numerous proteins into their environment for growth and survival under complex and ever-changing conditions. The highly different characteristics of secreted proteins pose major challenges to the cellular protein export machinery and, accordingly, different pathways have evolved. While the main secretion (Sec) pathway transports proteins in an unfolded state, the twin-arginine translocation (Tat) pathway transports folded proteins. To date, these pathways were believed to act in strictly independent ways. Here, we have employed proteogenomics to investigate the secretion mechanism of the esterase LipA of *Bacillus subtilis*, using a serendipitously obtained hyper-producing strain. While LipA is secreted Sec-dependently under standard conditions, hyper-produced LipA is secreted predominantly Tat-dependently via an unprecedented overflow mechanism. Two previously identified *B. subtilis* Tat substrates, PhoD and YwbN, require each a distinct Tat translocase for secretion. In contrast, hyper-produced LipA is transported by both Tat translocases of *B. subtilis*, showing that they have distinct but overlapping specificities. The identified overflow secretion mechanism for LipA focuses interest on the possibility that secretion pathway choice can be determined by environmental and intracellular conditions. This may provide an explanation for the previous observation that many Sec-dependently transported proteins have potential twin-arginine signal peptides for export via the Tat pathway.

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INTRODUCTION

Microorganisms secrete numerous enzymes into their extracellular milieu, enabling them to degrade a wide variety of macromolecular substrates and survive in complex and continuously changing environments [1-5]. Bacterial protein secretion is thus a highly relevant topic in both fundamental molecular microbiological research as well as in applied research, especially since many of the secreted enzymes have (potential) applications in industry. The Gram-positive bacterium *Bacillus subtilis* serves as an excellent model organism to study protein secretion for several reasons. *B. subtilis* lacks an outer membrane, which retains many proteins in the periplasm of Gram-negative bacteria, such as *Escherichia coli*. Accordingly, the majority of *B. subtilis* proteins that are transported across the cytoplasmic membrane end up in the extracellular milieu of this bacterium [4-6]. This property makes *B. subtilis* an extremely attractive organism to investigate the total flow of proteins from the cell to the environment by proteomic techniques [5, 7-10]. Furthermore, the availability of the complete genome sequence [11], readily available strains with mutations in nearly all of the ~4100 genes [12], industrial application of many native secreted proteins [13] and numerous useful techniques for gene cloning and expression [14, 15] have made *B. subtilis* the major paradigm for research into protein secretion by Gram-positive bacteria.

Previous studies have described the composition of the so-called secretome of *B. subtilis* which, by definition, includes all secreted proteins *plus* the protein secretion machinery itself [4, 5, 9]. The results of proteomics studies were complemented by secretome predictions based on the presence of specific targeting sequences that many secreted proteins have in common [16-18]. Furthermore, it was established that at least four distinct pathways for protein export from the cytoplasm are active [5, 7]. The majority of secreted proteins appear to be exported *via* the general secretory (Sec) pathway. A limited number of other proteins are transported *via* the twin-arginine translocation (Tat) pathway, the pseudopilin export pathway for competence development (Com pathway), and pathways using ABC transporters dedicated to the secretion of bacteriocins and pheromones (Bacteriocin pathway).

The Tat pathway has attracted particular interest due to its capability of translocating fully folded proteins across biological membranes. Active Tat translocases have been identified in Gram-negative and Gram-positive bacteria, as well as in archaea and chloroplasts [19-26]. These

translocases have been named after the conserved twin-arginine (RR) motif found in the signal peptides of proteins that they transport. This motif is located in the N-terminal region of twin-arginine signal peptides and its consensus sequence was originally defined as (S/T)-R-R-x-F-L-K [27-29]. The core sequence of functional RR-motifs seems to be R/K-R-x-#-# (where # is a hydrophobic residue; [30]. The currently best-characterized Tat system is that of *E. coli*, which consists of three integral membrane proteins known as TatA, TatB and TatC. The current opinion within the field is that TatB and TatC serve in the initial RR-signal peptide reception, while TatB and TatC in complex with multiple TatA components form a protein-conducting channel [31-33). Interestingly, *B. subtilis*, as well as most other Gram-positive bacteria, do not possess a TatB homologue [34-36]. Instead, these organisms contain minimal TatAC translocases in which a bifunctional TatA subunit seems to perform not only the role of TatA, but also that of TatB [37, 38]. Notably, *B. subtilis* contains three TatA proteins (named TatAc, TatAd and TatAy) and two TatC proteins (named TatCd and TatCy) [34]. Previous studies have shown that these Tat proteins form two distinct TatAC translocases (named TatAdCd and TatAyCy) for the export of specific substrates. The TatAdCd translocase is required for secretion of the phosphodiesterase PhoD [34], while the TatAyCy translocase is required for secretion of a Dyp-type peroxidase called YwbN [35]. A specific role for TatAc in translocation has so far not been demonstrated. In accordance with their coupled functions, the *tatAd* and *tatCd* genes are organized in an operon, as are the *tatAy* and *tatCy* genes.

Previous estimates have indicated that of ~200 predicted exported proteins with cleavable signal peptides in *B. subtilis*, 69 could potentially use the Tat pathway as their signal peptides carry RR- or KR-motifs that conform to the core consensus [39]. However, PhoD and YwbN have so far remained the only experimentally confirmed proteins that are secreted through this route. The signal peptides of two additional proteins, QcrA and YkuE were recently shown to direct secretion of an agarase *via* the Tat system of *Streptomyces coelicolor* when fused to this protein [40]. These findings have led to the conclusion that the Tat system of *B. subtilis* is highly selective [39]. A so far unresolved question concerned the Tat-dependent or Tat-independent secretion of the esterase LipA. The signal sequence of LipA conforms well to the most stringent criteria that are currently available for the prediction of RR-signal peptides, and a moderate effect on LipA secretion was observed in a *tatCd* deletion strain [39]. However, efficient Tat-independent LipA secretion could be

demonstrated in a *B. subtilis* mutant lacking all five *tat* genes, and the secretion of this protein was shown to depend on the motor component SecA of the Sec-dependent secretion pathway [39].

In the present studies, we have reinvestigated the Sec- or Tat-dependent secretion of LipA employing a serendipitously obtained mutant strain that hyper-secretes LipA. Using a proteo-genomics approach, we show that under conditions of massive LipA overproduction, this protein is secreted predominantly Tat-dependently.

To our knowledge, this is the first reported case of an authentic, non-recombinant, secretory protein that can employ both the Sec and Tat pathways for export from the cytoplasm in a Gram-positive bacterium.

RESULTS

The LipA hyper-producing strain XdsbA*.

In a recent study, we compared the thiol-disulfide oxidoreductase activities of the *S. aureus* lipoprotein DsbA and the homologous *B. subtilis* membrane protein BdbD [41]. For this purpose, we expressed the *S. aureus* DsbA with a xylose-inducible promoter from a cassette that was integrated into the *amyE* locus of the *B. subtilis* chromosome. The resulting strain was named XdsbA. Unexpectedly, when the secreted proteins of different XdsbA isolates were analyzed by proteomics, one of them was found to display a remarkably high secretion of LipA, while all other XdsbA isolates produced LipA at wild-type levels. Immunoblotting with specific antibodies against LipA clearly confirmed that this strain, annotated as XdsbA*, produced massively increased levels of LipA compared to the regular XdsbA strain (Fig. 1). In fact, the production of LipA was so high that the LipA produced by the parental strain 168, the regular XdsbA strain and a control strain with the empty XTC cassette is barely detectable on the same blot due to the strong signal obtained for the LipA overproduced by the XdsbA* strain.

Interestingly, the level of LipA hyper-secretion by the XdsbA* strain was significantly increased when xylose was added to the growth medium (Fig. 1). For this reason, in all further experiments cells were grown in the presence of 1% xylose. Under these conditions, the processing of pre-DsbA to mature DsbA was reduced (Fig. 1), suggesting that LipA hyper-production interferes to some extent with the Sec-dependent pre-DsbA export and/or processing by the lipoprotein-specific signal peptidase II.

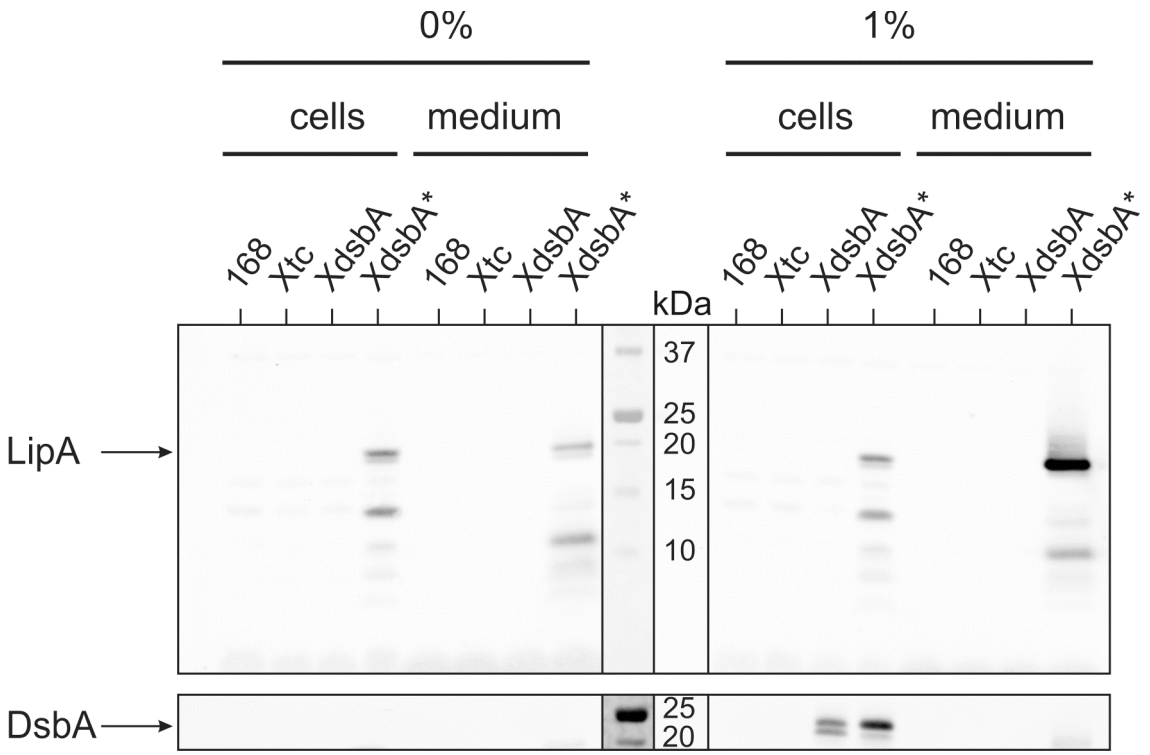

Figure 1. LipA secretion by the XdsbA* mutant. *B. subtilis* strains 168, Xtc, XdsbA and XdsbA* were grown overnight in LB medium in the absence (0%) or presence (1%) of xylose. Cells were separated from the growth medium by centrifugation and the presence of LipA or DsbA in both fractions was investigated by SDS-PAGE and subsequent Western blotting with specific antibodies against LipA or DsbA, respectively. Note that, due to the high concentrations of LipA, medium fractions were not concentrated. Arrows indicate the position of LipA. Bands with a higher mobility on SDS-PAGE are presumably degradation products of LipA. Molecular weight markers are indicated (kDa).

LipA hyper-production by the XdsbA* strain is caused by strongly increased *lipA* transcription.

To pinpoint the possible cause of the hyper-production of LipA by the XdsbA* strain, we first verified the correct integration of the XdsbA cassette in the *amyE* locus by PCR and sequencing (data not shown). This confirmed that no chromosomal rearrangement had occurred, at least with respect to the *amyE* locus and the integrated XdsbA cassette. Next, we investigated whether the hyper-production of LipA might be related to possible differences in the expression of the *dsbA* gene as encoded by the XdsbA cassette. To this end, we performed Northern blotting experiments using mRNA extracted from the XdsbA and XdsbA* strains, using a specific probe complementary to the *dsbA* mRNA. mRNA from the Xtc strain was used as a negative control. The results of this analysis, shown in Figure 2A, confirmed that the *S. aureus dsbA* gene was equally well transcribed in both strains, whereas no *dsbA* transcript was detectable in the strain containing the

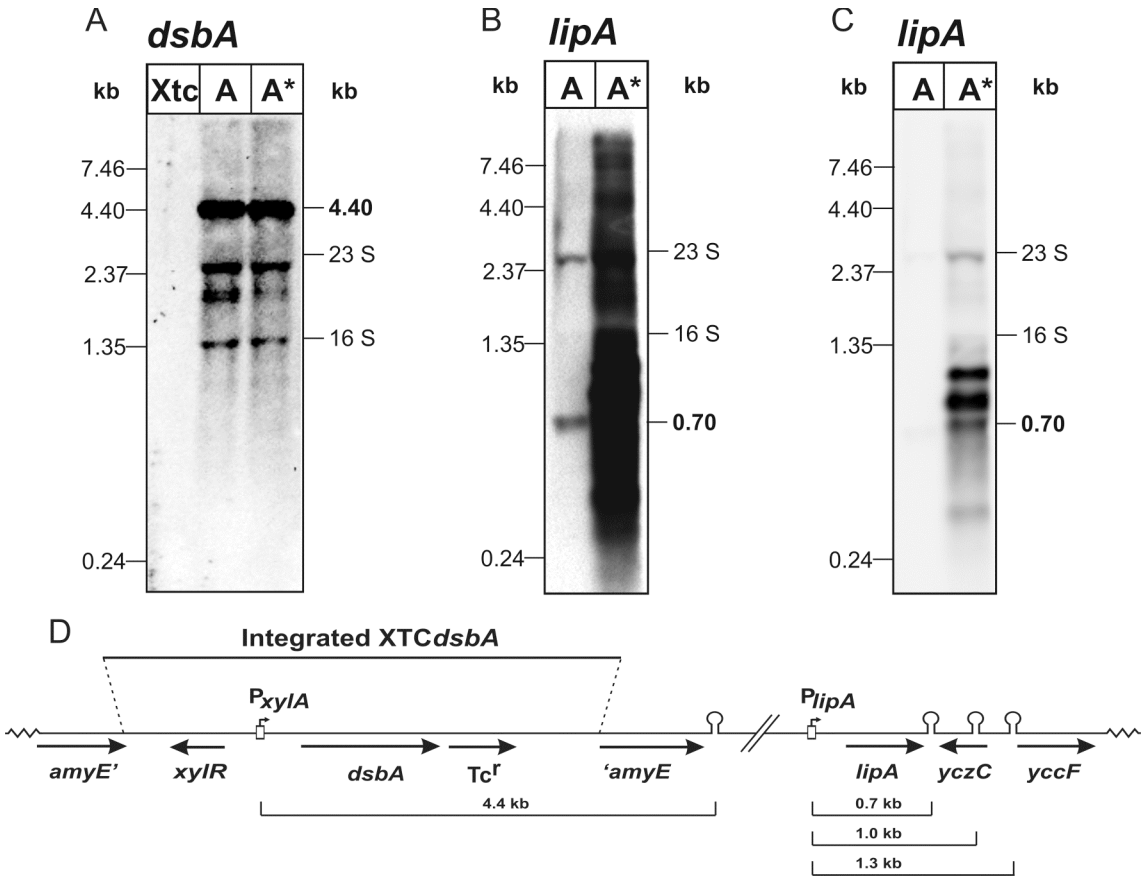

Figure 2. Northern blotting analysis of *dsbA* and *lipA* transcription. (A-C) RNA isolation and Northern blotting were performed as described in the Materials and Methods section. For each sample, 5 µg of RNA per lane were loaded. The lanes are labeled as follows: Xtc, RNA of *B. subtilis* strain Xtc (contains the empty XTC cassette in *amyE*); A, RNA from *B. subtilis* strain XdsbA (integrated XdsbA cassette for expression of *S. aureus dsbA*); A*, RNA from *B. subtilis* strain XdsbA* (integrated XdsbA cassette for expression of *S. aureus dsbA* and harboring an unknown mutation causing hyper-expression of *lipA*). The RNA probes used for the particular hybridizations are indicated above the images and are complementary to the first 600 bases of the coding regions of either *dsbA* or *lipA*. Molecular markers and the positions of the 16S and 23S rRNA are indicated. Panel C is a shorter exposure of the same chemiluminograph that is shown in panel B.

(D) Schematic representation of the chromosomal *amyE* and *lipA* regions of the XdsbA and XdsbA* strains. Both strains contain the X*dsbA* cassette integrated in the *amyE* gene via a double crossover recombination event. This cassette encodes the mature *dsbA* gene from *S. aureus* fused to the ribosomal binding site and signal sequence of *mntA* from *B. subtilis*. The hybrid *dsbA* gene is transcribed from a xylose-inducible promoter (P_{xylA}), resulting in a transcript of 4.4 kb (panel A) that is terminated downstream of *amyE*. *xylR*, gene specifying the XylR repressor protein; *amyE'*, 3' truncated *amyE* gene; '*amyE*, 5' truncated *amyE* gene; Tc^R, tetracycline resistance marker. Three predicted terminators are indicated downstream of *lipA*. These terminate transcription from the *lipA* promoter (P_{lipA}), which results in transcripts of 0,7 kb, 1.0 kb and 1.3 kb (panel C). Please note that *lipA* is annotated as *lip* in the SubtiList data base (http://genolist.pasteur.fr/SubtiList).

empty XTC expression cassette. Notably, the band of ~4.4 kb representing the *dsbA*-specific mRNA was larger than expected on the basis of the *dsbA* gene alone (666 nucleotides). This indicates that the transcription of *dsbA* is terminated downstream of the *amyE* gene (Fig. 2D). As the level of *dsbA* transcription was comparable in the XdsbA and XdsbA* strains, we conclude that the observed difference in the production of LipA by these strains could not be attributed to indirect effects relating to the expression of *dsbA*. This view is in agreement with the fact that comparable amounts of DsbA (precursor *plus* mature) were produced in the XdsbA and XdsbA* strains when grown in the presence of xylose (Fig. 1). Thus, it seemed that LipA hyper-production was related to increased *lipA* gene expression.

To assess whether the hyper-production of LipA did originate from increased *lipA* expression on the mRNA level, we performed a Northern blotting analysis using a specific *lipA* probe. As shown by the results in Figure 2 (B and C), the amount of *lipA*-specific mRNA was strongly increased in the XdsbA* strain. In order to resolve the different individual mRNA species, two images of the same Northern Blot exposed for different periods of time are shown. The longer exposure (Fig. 2B) reveals the presence of a 0.7 kb *lipA*-specific transcript in the XdsbA strain, representing the original monocistronic *lipA* transcript. This transcript seems to be terminated at the distinct transcriptional terminator, which can be derived from the DNA sequence immediately downstream of *lipA*. In the lane representing the XdsbA* strain only a dense black smear is visible due to the large amounts of *lipA*-specific mRNA in this strain. The shorter exposure reveals only very faint signals in the XdsbA lane, but allows detection of three *lipA*-specific mRNA species in the XdsbA* strain (Fig. 2C). The smallest among these again corresponds to the 0.7 kb mRNA, which is also detectable in the XdsbA strain. In addition, there are two *lipA*-specific mRNA species, which are not detectable in the XdsbA strain. The estimated sizes of these transcripts correspond to ~1.0 and ~1.3 kb. These additional bands most probably represent read-through transcripts that are not terminated at the transcriptional terminator immediately downstream of *lipA*, but somewhat further downstream. Indeed, a systematic search for stem-loop structures in the region downstream of *lipA*, using the program mfold [52, 53] (http://helix.nih.gov/docs/gcg/mfold.html), revealed two distinct stem-loops: the first one located within the coding region of *yczC* (the gene downstream of *lipA* which is transcribed in the opposite direction) and the second located immediately upstream of *yccF*, within the promoter region of this

gene. Both of these structures could function as transcriptional terminators or act as a 3`-stabilizer against 3`-exonucleases. The derived sizes of the mRNAs, which are predicted to terminate at these two sites, amount to 1.0 kb and 1.3 kb, therefore matching the sizes of the bands detected by Northern blotting (Fig 2D). Experimental confirmation of this *in silico* analysis was obtained by Northern blotting experiments using the D1 and D2 probes that were designed to hybridize to the two regions between the three stem-loops downstream of *lipA*. Using these probes, the 1.0 kb and 1.3 kb mRNAs were indeed detected again (results not shown), demonstrating that all the different *lipA*-specific mRNA species up-regulated in XdsbA* originate at the original *lipA* promoter. Taken together, these results demonstrate that the LipA hyper-production by the XdsbA* strain is correlated with strongly increased amounts of *lipA*-specific mRNAs, most probably due to strongly enhanced transcription of the *lipA* gene.

Sequencing of the 0.5 kb *lipA* promoter regions in the XdsbA and XdsbA* strains revealed that there are no alterations in the promoter sequence that could be responsible for the increased *lipA* transcription in the XdsbA* strain. Interestingly, when we transformed the parental strain *B. subtilis* 168 with genomic DNA of the XdsbA* strain and selected for tetracycline resistant transformants that contained the XdsbA cassette integrated in *amyE*, we observed that about 15% of these clones displayed the LipA hyper-production phenotype. This shows that the genomic alteration in the XdsbA* strain that is responsible for LipA hyper-production is located close to the *amyE* region, probably within a range of ~10-15 kb upstream or downstream of *amyE*. By contrast, no LipA hyper-producers were identified when the 168 strain was transformed with chromosomal DNA from the XdsbA strain. Taken together these findings show that the observed hyper-production of LipA in the XdsbA* strain is due to de-repressed or hyper-activated transcription of the *lipA* gene.

Transcriptome analysis of the LipA hyper-producing XdsbA* strain.

To determine which other genes had altered expression levels in the XdsbA* strain, we performed a genome-wide transcriptional analysis with microarrays. To distinguish between effects of the expression of *dsbA* and the unknown mutation causing *lipA* hyper-expression, two different sets of comparisons were carried out. For identifying the effects of the unknown mutation in the XdsbA* strain, we compared RNA from the XdsbA* and XdsbA strains. For visualization of the effects of *dsbA* expression we compared the RNA of the XdsbA strain with that of the

Xtc control strain, harboring the empty integrated XTC expression cassette. The statistically significant effects are listed in Table I. The first striking observation was that there are only a few genes up- or down-regulated in the XdsbA* strain compared to the XdsbA strain (six genes up-regulated and nine genes down-regulated). None of the affected genes however reside within the ~20-30 kb range around *amyE* gene, which indicates that the changed transcription of these genes is not likely the primary cause of the increased *lipA* transcription. Another striking observation is that most of the genes that are significantly up- or down-regulated in the XdsbA strain are inversely regulated in the XdsbA* strain (five of the seven down-regulated genes in XdsbA are up-regulated in XdsbA*; all seven up-regulated genes in XdsbA are down-regulated in XdsbA*; Table I). For example, the *manRPA* gene cluster is down-regulated in the XdsbA strain, but up-regulated again in the XdsbA* strain. Conversely, the *yddJ-rapI-phrI-yddM* and *ydcM-ydcN-ydcO* gene clusters are up-regulated in the XdsbA strain, but down-regulated in the XdsbA* strain.

Only two genes that are down-regulated in the XdsbA strain (*yrhG* and *yrhE*) are not up-regulated again in the XdsbA* strain, one gene up-regulated in the XdsbA* strain (*lipA*) is not affected in the XdsbA strain, and two genes down-regulated in the XdsbA* strain (*yonBC*) are not affected in the XdsbA strain. Thus, the effects of *dsbA* expression on the expression of other genes in *B. subtilis* seem to be largely reversed in the XdsbA* strain. This implies that the unidentified mutation in the XdsbA* strain that causes *lipA* hyper-expression is a mutation that counteracts certain possibly detrimental effects of DsbA production in *B. subtilis*.

An important outcome of the transcriptome analyses is that the mutation causing LipA hyper-secretion by the XdsbA* strain has no detectable effect on any of the known genes for secretion machinery components. This includes the genes for proteins that target secretory precursor proteins to the protein export machinery (*e.g. ffh*, *ftsY* and *csaA*), genes for protein translocation across the membrane (*e.g.* the *sec* and *tat* genes; see Table I), and genes for post-translocational modification and folding of secretory proteins (e.g. the *bdb*, *sip*, *skf* and *prsA* genes). Thus, it can be concluded that the mutation in the XdsbA* strain causing LipA hyper-production acts solely at the *lipA* transcriptional level. The XdsbA* strain can therefore be used to study the mechanism of LipA secretion under conditions of hyper-production.

Table I. Microarray analysis

To monitor the genome-wide effects on transcription of an unidentified mutation in the XdsbA* strain causing LipA hyper-production, or the expression of *S. aureus dsbA* in the XdsbA strain, microarray analyses were performed. Preparation of total RNA, cDNA synthesis, labelling, and DNA microarray hybridization and analysis were performed as indicated under Materials and Methods. The RNA samples obtained from three independent cultivations of the XdsbA*, XdsbA and Xtc strains were used for independent cDNA synthesis and competitive DNA array hybridization in two groups: XdsbA* *versus* XsbA, and XdsbA *versus* Xtc. The comparison of the XdsbA* and XsbA RNAs shows genome-wide effects of the mutation causing LipA hyper-production, the comparison of XdsbA and Xtc RNAs shows the effects of *dsbA* expression. Significantly up- or down-regulated genes, as displayed in this Table, were considered as such when the mRNA abundance between two compared strains had Cyber-T Bayesian *P* values of < 0.001 and the individual fold change was at least 2. To show the absence of effects of LipA hyper-production on *tat* gene expression, the results obtained for the five *B. subtilis tat* genes are included in this Table. Results are sorted by the average RNA abundance levels in XdsbA*. Average changes of over two-fold are marked in bold.

		Ratio of XdsbA* over XdsbA				Ratio of XdsbA over Xtc			
Gene	Acc. nr.[1]	I	II	III	Average	I	II	III	Average
lipA	BG10679	152,95	118,15	112,57	**127,89**	0,75	0,91	0,85	0,84
manP	BG13176	9,58	2,32	5,47	**5,79**	0,07	0,50	0,32	**0,30**
manA	BG13177	6,17	2,14	3,51	**3,94**	0,14	0,79	0,34	**0,42**
manR	BG13175	2,69	2,03	6,42	**3,71**	0,28	0,83	0,25	**0,45**
yxiE	BG11134	1,00	1,47	5,00	**2,49**	0,45	0,39	0,35	**0,40**
bglH	BG10935	1,47	1,28	3,78	**2,17**	0,42	0,20	0,36	**0,33**
yrhG	BG12296	1,53	2,43	0,87	1,61	0,22	0,29	0,36	**0,29**
yrhE	BG12294	1,39	0,73	1,02	1,05	0,39	0,28	0,24	**0,30**
ydcO	BG12102	0,54	0,41	0,43	**0,46**	2,14	2,68	2,48	**2,43**
yonC	BG13616	0,36	0,28	0,48	**0,37**	0,74	1,98	0,36	1,02
yonB	BG13615	0,35	0,23	0,38	**0,32**	0,65	2,32	0,38	1,12
rapI	BG12119	0,29	0,27	0,31	**0,29**	3,49	2,64	2,89	**3,01**
phrI	BG12645	0,30	0,23	0,28	**0,27**	3,04	3,49	3,59	**3,38**
yddM	BG12120	0,18	0,16	0,14	**0,16**	9,89	11,04	7,60	**9,51**
yddJ	BG12117	0,13	0,16	0,13	**0,14**	5,65	6,03	7,83	**6,50**
ydcM	BG12100	0,05	0,08	0,06	**0,07**	15,79	19,48	9,31	**14,86**
ydcN	BG12101	0,05	0,04	0,05	**0,04**	19,37	19,16	22,48	**20,34**
tatAd	BG12777	1,13	1,16	1,00	1,09	1,41	0,95	1,09	1,15
tatAy	BG12206	1,37	0,89	1,01	1,09	1,03	0,89	1,09	1,00
tatAc	BG13464	1,37	1,25	1,17	1,26	1,35	1,28	1,14	1,26
tatCd	BG11175	0,89	0,99	1,05	0,98	1,19	1,04	0,97	1,07
tatCy	BG12207	0,95	1,27	0,87	1,03	0,99	0,91	0,81	0,90
mntA[2]	BG13851	1,36	0,75	1,13	1,08	19,97	9,28	29,52	**19,59**

[1]Accession numbers were derived from the subtilist database (http://genolist.pasteur.fr/SubtiList).
[2]*mntA* appears to be up-regulated in the *dsbA* expressing strain, but this is due to the fact that the *mntA* signal sequence was fused to the *dsbA* gene for efficient targeting of DsbA to the membrane of *B. subtilis*. This value thus reflects the expression of the *S. aureus dsbA* gene rather than an altered expression of the *B. subtilis mntA* gene.

The XdsbA* strain secretes reduced amounts of the Tat-dependent PhoD and YwbN proteins.

To obtain insights whether the massive overproduction of LipA interferes with the secretion of other proteins into the growth medium, we performed extensive exoproteome analyses. As a first approach, cells were grown to the post-exponential growth phase in the rich LB medium because, under these conditions, the largest numbers of secreted proteins have been identified previously [7]. Next, the extracellular proteins of the XdsbA* strain and the parental strain 168 were separated by two-dimensional gel electrophoresis (2D PAGE) and compared by dual channel imaging. The results revealed that apart from the strongly increased secretion of LipA, the extracellular proteome of the XdsbA* strain was virtually identical to that of the parental strain 168 (Fig. 3). This showed that neither the hyper-secretion of LipA, nor the production of *S. aureus* DsbA had any secondary effects on the composition of the extracellular proteome, at least when cells were grown in a rich medium. However, not all genes for secretory proteins are expressed when cells are grown in rich media such as LB [5]. Therefore, we also deployed two-dimensional gel electrophoresis to study the extracellular proteome of *B. subtilis* cells under phosphate-limiting conditions. Under these growth conditions, *B. subtilis* will secrete a set of phosphate starvation-induced proteins, which are not produced by cells grown in rich media [8]. One of these proteins is the Tat-dependently secreted protein PhoD [34]. Figure 4 displays the extracellular proteomes of the XdsbA* and 168 strains grown under phosphate-limiting conditions. These results showed that LipA was also hyper-secreted by the XdsbA* strain under conditions of phosphate limitation. Interestingly though, the level of PhoD secretion was strongly reduced in the XdsbA* strain compared to the secretion of all other proteins that are secreted via the Sec pathway, such as PhoB (compare the intensities of the PhoD spots to the intensities of other spots in Figure 4). This suggested that the hyper-secretion of LipA somehow interfered specifically with that of PhoD, possibly through a competition for translocation *via* the same Tat translocase.

To establish whether the secretion of LipA interfered specifically with PhoD secretion, or also with that of the second known Tat substrate of *B. subtilis*, YwbN, we performed Western blotting experiments. Since secretion of the authentic YwbN is not detectable during standard growth conditions, a plasmid-encoded Myc-tagged derivative of YwbN was used for this purpose. Next, we compared the secretion levels of YwbN-Myc in the presence or absence of LipA hyper-secretion.

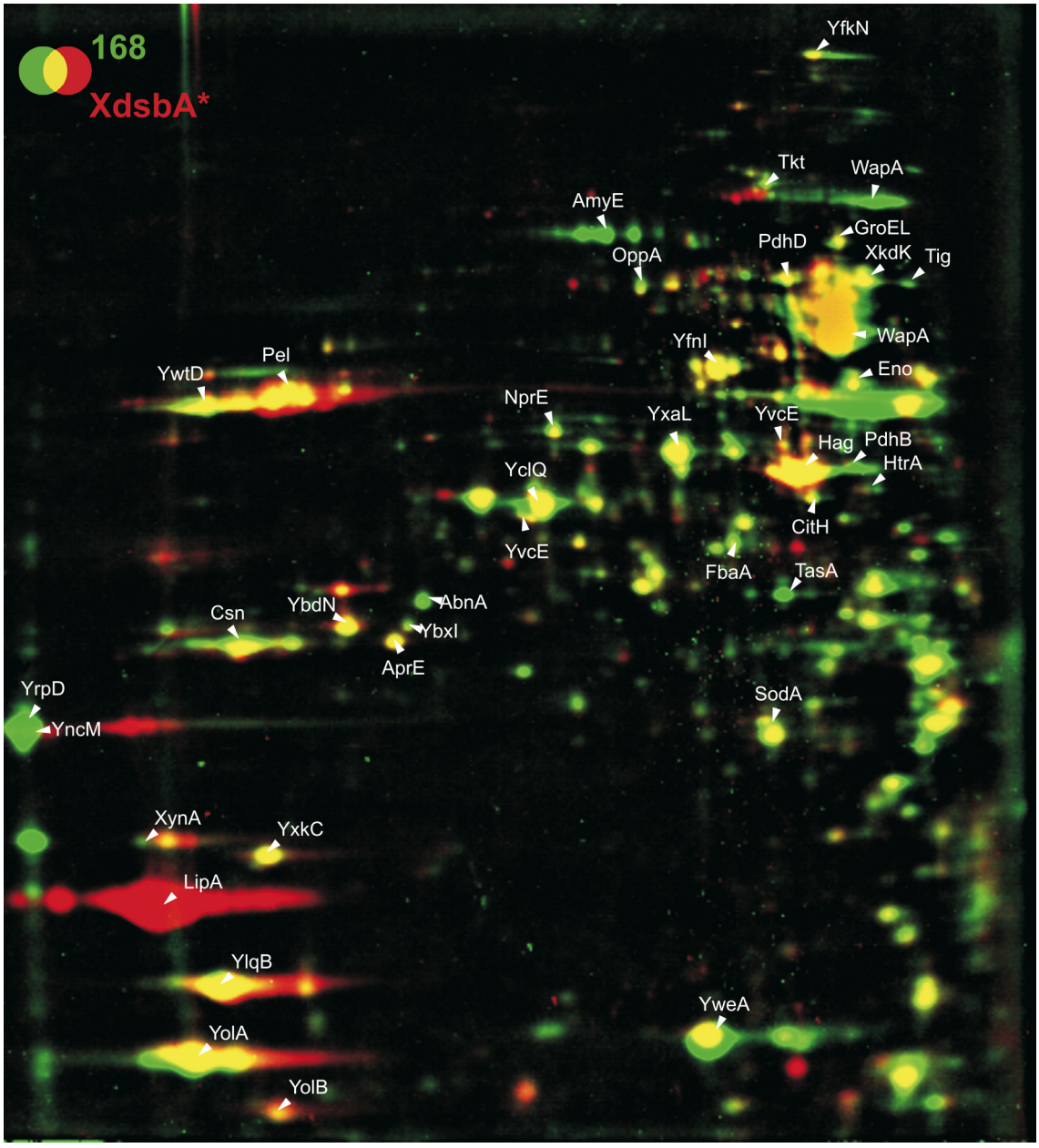

Figure 3. The extracellular proteome of *B. subtilis* XdsbA* grown in LB medium. Cells of *B. subtilis* XdsbA* and the parental strain 168 were grown in LB medium in the presence of 1% xylose and extracellular proteins were collected 1 h after entry into the stationary phase. Secreted proteins were analyzed by 2D PAGE and dual channel imaging as indicated under Material and Methods. Green protein spots are predominantly present in the master image of the extracellular proteins of *B. subtilis* 168; red protein spots are predominantly present in the image of the extracellular proteins of the *B. subtilis* XdsbA* strain; and yellow protein spots are present at similar amounts in both images. The presented picture was obtained by dual channel imaging of two representative warped images. The names of proteins previously identified by MALDI-TOF mass spectrometry are indicated [7, 9]. Although several green and red spots are present in the image, only the amount of LipA was significantly changed (increased) in the exoproteome of the XdsbA* strain. Note that the XdsbA* strain does not produce AmyE due to the insertion of the XdsbA cassette into the *amyE* gene.

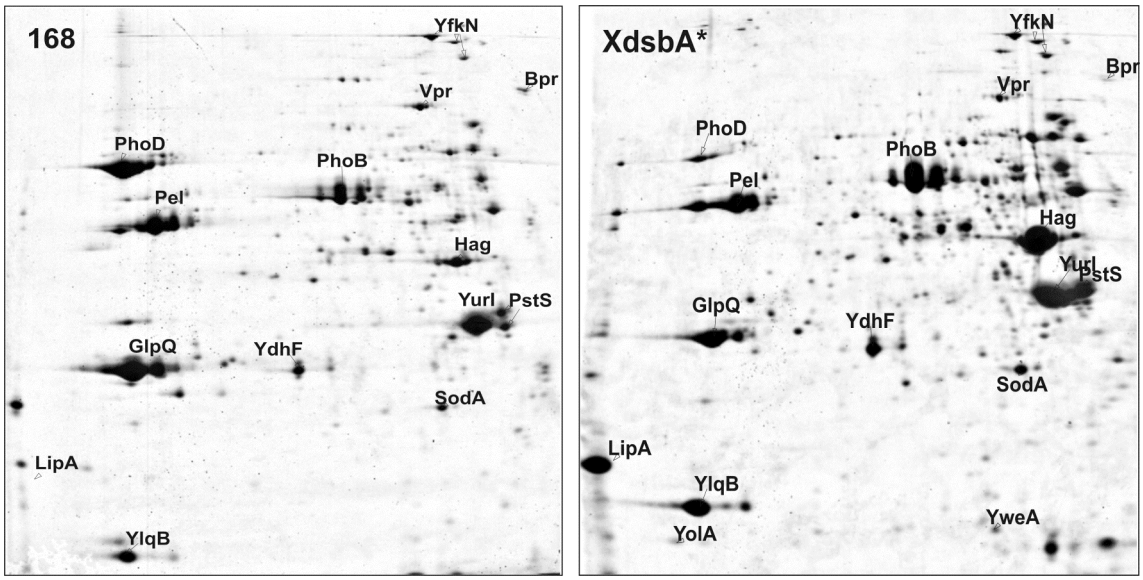

Figure 4. The extracellular proteomes of *B. subtilis* XdsbA* and 168 under phosphate-limiting conditions. *B. subtilis* XdsbA* and 168 were grown under conditions of phosphate starvation as previously described [8, 34]. Secreted proteins were analyzed by 2D PAGE as indicated under Material and Methods. The names of proteins previously identified by MALDI-TOF mass spectrometry are indicated [8, 34]. Note that the XdsbA* gel was loaded with slightly more protein than the 168 gel and that direct comparison of spot intensities between the two gels is not possible. Comparison of spot intensities per gel reveals that, in the XdsbA* strain, PhoD is secreted at reduced levels relative to other abundantly secreted proteins such as GlpQ, Hag, Pel, PhoB, PstS, and YurI.

Indeed, Figure 5 shows that the LipA hyper-secreting strain XdsbA* secretes about 2- to 2.5-fold reduced amounts of YwbN-Myc compared to the XdsbA or Xtc control strains as determined by densitometric image analysis. In addition, the cellular levels of YwbN-Myc are somewhat (about 1.5- to 2.0-fold) higher in the XdsbA* strain than in the control strains. These results therefore suggest that the hyper-produced LipA interferes with the secretion of YwbN, as was shown above for PhoD. Taken together, these findings suggest that the hyper-produced LipA competes with both PhoD and YwbN for secretion *via* the Tat pathway, which would imply that LipA can interact with both the TatAdCd and the TatAyCy translocases.

Secretion of hyper-produced LipA is predominantly Tat-dependent.

To test whether the hyper-secretion of LipA might depend to some extent on a functional Tat machinery, we deleted *tat* genes from the XdsbA* strain. While the removal of either the *tatAd-tatCd* or *tatAy-tatCy* genes from the XdsbA* background had moderately reducing or stimulating effects on the secretion of hyper-produced LipA, the removal of all four of these *tat*

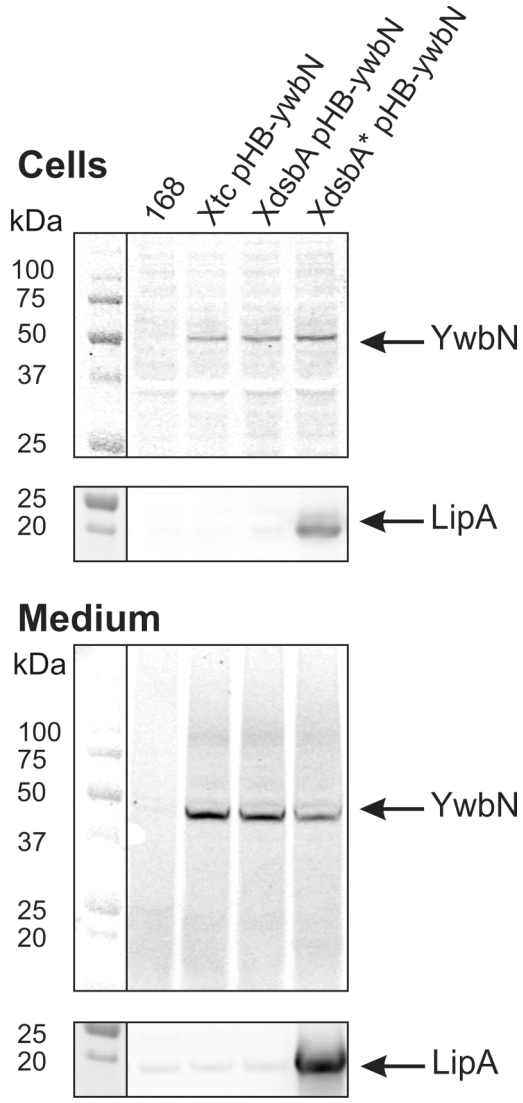

Figure 5. Secretion of YwbN-Myc by *B. subtilis* XdsbA*. Cells of *B. subtilis* strains 168, Xtc pHB-ywbN-myc, XdsbA pHB-ywbN-myc and XdsbA* pHB-ywbN-myc were grown overnight in LB in the presence of 1% xylose. To detect YwbN-Myc, cells were separated from the medium by centrifugation. Proteins in the medium were concentrated 20-fold upon precipitation with trichloroacetic acid (TCA) and samples for SDS-PAGE were prepared as described previously [34]. Western blotting was performed with monoclonal antibodies against the Myc-tag or polyclonal antibodies against LipA. The positions of YwbN and LipA are indicated by arrows. Molecular weight markers are indicated (kDa).

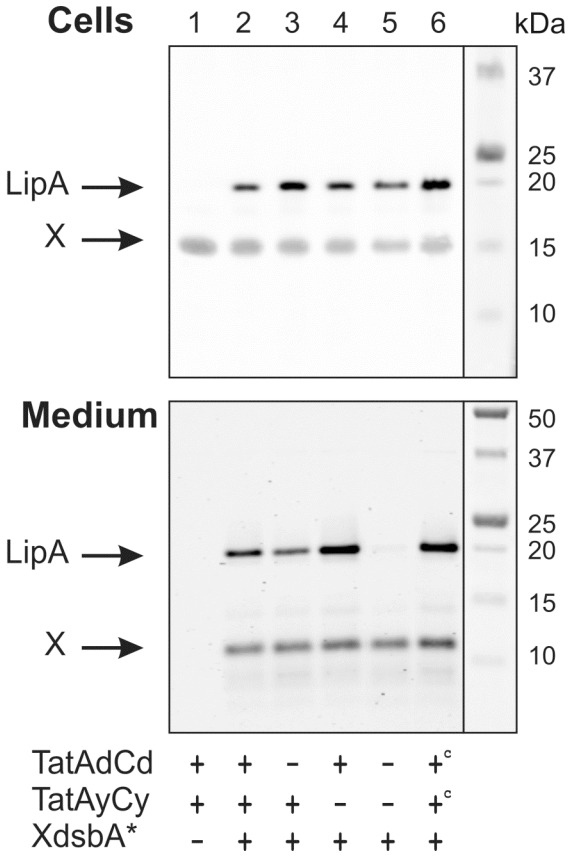

Figure 6. Tat-dependent secretion of hyper-produced LipA. Cells of *B. subtilis* strains 168 (1), XdsbA* (2), XdsbA* ΔtatAdCd (3), XdsbA* ΔtatAyCy (4), XdsbA* ΔtatAyCy ΔtatAdCd (5), and XdsbA* ΔtatAyCy ΔtatAdCd pHB-tatAdCd pCACy (6) were grown overnight in LB in the presence of 1% xylose. Three clones of each strain were inoculated in separate cultures and combined after growth. Cells were separated from media and the presence of LipA in these fractions was investigated by SDS-PAGE and subsequent Western blotting with specific antibodies against LipA. Arrows indicate the positions of LipA and additional bands (X) that cross-react with the LipA antibodies. Molecular weight markers are indicated. C, marks the complementation of strain XdsbA* ΔtatAyCy ΔtatAdCd with plasmids pHB-tatAdCd and pCACy carrying the *tat* genes that have been deleted from the chromosome. Molecular weight markers are indicated (kDa).

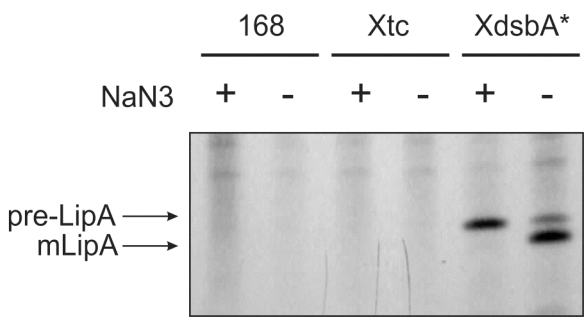

Figure 7. Processing of hyper-produced pre-LipA. Processing of pre-LipA to the mature form was analyzed in *B. subtilis* strains 168, Xtc and XdsbA*. Cells were pulse labeled for 1 min with [^{35}S] methionine/cysteine as described before [39, 50] in the presence of 1% xylose. When required, the SecA translocation inhibitor, sodium azide was added 5 min prior to labeling at 1.5 mM final concentration (+ NaN_3). Next, LipA specific antibodies were used for immunoprecipitation of LipA. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by fluorography.

genes caused a strongly reduced secretion of the hyper-produced LipA (Fig. 6). These results are reminiscent of earlier observations concerning PhoD secretion, which was impaired in the absence of TatCd, but slightly increased in the absence of TatAy, indicating some interference between the two Tat pathways of *B. subtilis*. It should be noted that the XdsbA* strain lacking TatAdCd and TatAyCy still secreted some LipA, but significantly less than the Tat proficient XdsbA* strain. Importantly, this defect in the secretion of hyper-produced LipA by the TatAdCd-TatAyCy deficient strain could be fully repaired by reintroduction of plasmid-borne copies of the *tatAd-tatCd* and *tatAy-tatCy* genes (Fig. 6). These results show that LipA hyper-secretion is Tat-dependent to a large extent and that this Tat-dependency relates to both the TatAdCd and TatAyCy translocases.

Previous studies had shown that LipA produced at wild-type levels was secreted in a Tat-independent manner, depending on the presence and activity of the SecA motor component of the Sec translocase [39]. To verify that the export of hyper-produced LipA was at least to some extent dependent of SecA, we performed pulse labeling experiments with the XdsbA* strain in the presence or absence of sodium azide. The SecA translocation ATPase inhibitor was added to the cells 5 min prior to labeling with [^{35}S] methionine/cysteine. Cells were labeled for 1 min and then any further incorporation of label was stopped by the addition of TCA. As shown by immunoprecipitation and subsequent fluorography, processing of pre-LipA to the mature form in the XdsbA* strain was sensitive to sodium azide (Fig. 7), indicating a SecA-dependency in pre-LipA processing. Notably, the LipA produced at wild-type levels by the Xtc and 168 control strains was not detectable, underscoring the massive degree of LipA overproduction by the XdsbA* strain. Taken together, our unprecedented observations show that LipA hyper-production leads to an overflow of this protein from the Sec-dependent pathway into the Tat pathway, and that the

LipA protein is intrinsically capable of leaving the cytoplasm *via* three translocases, namely Sec, TatAdCd and TatAyCy.

DISCUSSION

In the present studies we have used a proteogenomic approach to characterize the mechanism of hyper-secretion of the esterase LipA by a serendipitously obtained *B. subtilis* strain that over-expresses the *lipA* gene over a 100-fold. Proteomic analyses on this strain suggested that the LipA hyper-secretion by this strain interferes with the secretion of the two proteins for which Tat-dependent export from the cytoplasm has been documented, namely PhoD and YwbN. This suggested that the hyper-secretion of LipA might be Tat-dependent even though it had previously been shown that LipA produced at wild-type levels was Sec-dependently secreted. Indeed, secretion studies with *tat* mutant strains revealed a strong Tat-dependency of the hyper-secreted LipA. Remarkably, both the TatAdCd and the TatAyCy translocase are involved in LipA hyper-secretion. Indirect effects on the transcription of genes for known Tat or Sec pathway components were ruled out through the results of transcriptome analyses. It thus seems that an overflow mechanism directs the hyper-produced LipA from the Sec pathway into the Tat pathway, which is an unprecedented finding.

Protein secretion is an important topic in current molecular microbiological research related both to pathogens and organisms that are used as cell factories for biotechnological applications. Although many pathways for protein secretion have been described in considerable detail, the full complexity of these pathways and their interdependencies are not very well understood. Over the past decade, we have employed proteomics technology to map the different pathways of the secretome of *B. subtilis* [5, 7]. This has revealed a major role of the Sec pathway in protein secretion by this organism. Other pathways for protein secretion, such as the Tat pathway, were shown to serve highly selective purposes. Quite remarkably, merely two proteins (PhoD and YwbN) were shown to be exported Tat-dependently in *B. subtilis*, each of them requiring a distinct Tat translocase [35, 39]. This finding raised the intriguing question whether the Tat pathway in *B. subtilis* is really of minor importance, despite its intrinsic capacity to transport fully folded proteins [38], or whether it also serves particular other functions under certain conditions that had not yet been identified. In line with the latter idea was the finding that many

secreted *B. subtilis* proteins, like the esterase LipA, contain potential RR-motifs in their signal peptides. On the other hand however, our earlier proteomics studies clearly showed that all expressed proteins with potential RR-motifs (except PhoD and YwbN) were secreted Tat-independently, at least under the standard laboratory test conditions [5, 35, 39]. Our present studies have now identified one condition under which a normally Sec-dependently secreted protein with a potential RR-motif in its signal peptide can take a turn from the Sec pathway into the Tat pathway, namely the condition of hyper-secretion. Although this finding was unexpected, it is retrospectively perhaps not completely surprising since *B. subtilis* and related *Bacillus* species rely on the high-level secretion of degradative enzymes for their growth and survival in the soil and plant rhizosphere, for which they need to feed on decaying organic matter [54]. Under certain natural conditions, regulated hyper-secretion of degradative enzymes is therefore likely to occur, and the fact that a spontaneous mutation actually resulted in the hyper-secretion of LipA is fully consistent with this view. Thus, we believe that the current observations may have a broader significance for protein secretion by bacilli and other bacteria under natural conditions.

As yet, the exact location and nature of the mutation causing *lipA* hyper-expression from the original *lipA* promoter region is not known. It is however located in the vicinity of the *amyE* gene since frequent (~15%) co-transformation of the XdsbA cassette in the *amyE* gene and the mutation was observed. No obvious candidate genes for *trans*-acting factors in *lipA* expression located near the *amyE* gene could so far be identified. Clearly, sequence analysis identified no mutations in the upstream region of *lipA*, and we consider the distance between *lipA* and *amyE* (35 kb) too large to account for the frequent co-transformation of the XdsbA cassette in *amyE* with the mutation causing *lipA* overexpression. Transcriptome analyses of the XdsbA* strain did, unfortunately, not reveal potential locations for the unknown mutation since the transcription of none of the genes in the vicinity of *amyE* was altered. It thus seems most likely that a point mutation in a *trans*-acting transcriptional activator or repressor of *lipA* is responsible for the LipA hyper-production phenotype of the XdsbA* strain. Interestingly, our transcriptome analyses show that the unidentified mutation has by-and-large a counter-acting effect on the cellular response of *B. subtilis* to the expression of the *S. aureus dsbA* gene. This view is underscored by the fact that increasing the *dsbA* expression by the addition of xylose to the medium is followed by an increased production of LipA. Together, these findings suggest that expression of the *dsbA* gene is to some

extent stressful for the *B. subtilis* cell and that this stress is relieved by the mutation in the XdsbA* strain. However, we have so far not observed any phenotypes of the XdsbA strain that suggest any severely detrimental effects of DsbA production. In any case, the hyper-expression of *lipA* in our mutant would be a side-effect of the compensatory mutation in the XdsbA* strain as the expression of *lipA* was not affected by *dsbA* expression in the original XdsbA strain. It should be noted that the mutation leading to LipA hyper-production seems to occur infrequently since we observed this phenomenon only once so far. Although we do not know the precise nature of the mutation causing LipA hyper-production in the XdsbA* strain, we conclude from the transcriptome analyses that this mutant can be regarded as a *bona fide* overproduction strain with no side effects on the expression of secretion machinery components. In fact, this mutant does not even display a secretion stress response despite the hyper-production of LipA [9]. Thus, the XdsbA* strain can be used for studies on the LipA secretion mechanism, like any other strain overproducing a secretory protein.

Our proteomics analyses suggest that the hyper-produced LipA competes with PhoD and YwbN for secretion *via* the TatAdCd and TatAyCy translocases, respectively. These findings provided in fact the first incentive to investigate the Tat-dependency of LipA hyper-secretion, which was then confirmed by our experiments with multiple *tat* mutant strains hyper-producing LipA. Although a possible involvement of the Tat pathway in LipA secretion has been contemplated before [39], conclusive evidence for Tat-dependent LipA secretion could not be obtained until the LipA hyper-secreting XdsbA* strain became available. Importantly, the finding that LipA hyper-secretion depends on both Tat translocases of *B. subtilis* (*i.e.* TatAdCd and TatAyCy) is unprecedented and has not been reported for any other Tat substrate in *B. subtilis* or other organisms with duplicated Tat systems. This finding shows for the first time that the two *B. subtilis* Tat translocases do not have strictly separated specificities as was previously suggested [35, 36], but that their specificities overlap at least to some extent. It should be noted that we currently do not know what determines the specificity of Tat translocation pathway choice in *B. subtilis*. This may relate to the RR-sequence motifs in signal peptides, the nature and folding state of the mature protein, or all these factors together. In this respect, we cannot exclude the possibility that, under non-hyper-producing conditions, LipA is a preferred substrate for only one of the two *B. subtilis* Tat translocases. This is however hard to assess due to the predominantly Sec-dependent translocation of LipA under non-hyper-producing conditions, as well as the essentiality of the

Sec machinery for bacterial growth and life [39]. In the specific case of LipA hyper-production, we believe that the massive production of pre-LipA somehow saturates the Sec translocase for this protein. This would then lead to reduced LipA translocation rates and possibly folding of LipA in the cytoplasm. The folded pre-LipA would thus become a better substrate for the Tat machinery, which is known to accept mainly folded proteins. Alternatively, saturation of the Sec translocase might lead to a more effective recognition of the RR-motif in pre-LipA by the Tat machinery and, in this case, cytoplasmic folding of LipA might not be a strict prerequisite for Tat-dependent export. For example, unfolded DHFR can be translocated *via* the thylakoidal Tat system [55] and unstructured, small, hydrophilic proteins can be exported Tat-dependently in *E. coli* [56]. In any case, evidence for some degree of Sec pathway saturation by LipA hyper-production was indeed derived from the observation that the DsbA precursor accumulates in the XdsbA* strain, whereas this is not the case in the XdsbA strain. On the other hand, the secretion of the majority of proteins was not affected at the steady state level as was shown by proteomics. This is in fact consistent with the finding that LipA hyper-production did not interfere with cell growth and viability, which would have been the case if the Sec pathway had been jammed in a major way. Apparently, jamming of the Sec pathway is prevented by overflow of pre-LipA into the Tat pathway. Finally, our pulse labeling experiments suggest that the processing of newly synthesized hyper-produced pre-LipA is sensitive to sodium azide, indicating involvement of SecA in this precursor processing. This is consistent with our previous observations that LipA can be secreted Sec-dependently and with our present model that the observed Tat-dependency of hyper-produced LipA relates to an overflow mechanism from the Sec pathway into the Tat pathway. We do however not know the fate of the mature hyper-produced LipA that is detected by pulse labeling. In fact, it is quite conceivable that a substantial portion is degraded at the *trans* side of the membrane, as was previously shown for over-produced amylases that do not fold efficiently enough upon membrane translocation via the Sec pathway in an unfolded state [57, 58]. Notably, we can not completely exclude the possibility that sodium azide also affects the export of LipA via the *B. subtilis* Tat pathway. To our knowledge there are only two reported cases where the transport of Tat-dependent proteins was affected by sodium azide. These concern the Glucose-fructose oxidoreductase (GFOR) of the Gram-negative bacterium *Zymomonas mobilis* [59], and the 17-kD subunit of the photosynthetic O2-evolving complex (OE17) in intact chloroplasts [60]. It is

however not entirely clear whether these effects of sodium azide are direct or indirect effects.

In conclusion, our present findings show for the first time that certain secretory proteins, such as the esterase LipA of *B. subtilis*, can be exported both via the Sec and the Tat pathways depending on the conditions applied. To date, a re-routing of proteins from the Sec to the Tat pathway or *vice versa* has been achieved only artificially by replacing Sec-type signal peptides with RR-signal peptides and/or by modulating the folding conditions for the exported proteins so that they fold in the cytoplasm prior to membrane translocation. This has been worked out especially well in *E. coli* for re-routed export of the alkaline phosphatase PhoA and the maltose-binding protein MalE from the Sec pathway into the Tat pathway, and for the re-routed export of the ribose-binding protein RbsB from the Tat pathway into the Sec pathway [61-64].

Although the overflow mechanism as presented in our paper has not been documented before, we believe that it may be a more common, but so far overlooked, mechanism for the secretion of proteins that can fulfill the requirements for transport *via* both the Sec pathway (*i.e.* channel passage in an unfolded state) and the Tat pathway (*i.e.* folding prior to channel passage). It will be an important challenge for future studies to identify the environmental or cellular conditions that influence the usage of particular secretory pathways in order to obtain a full understanding of the biological processes that require an active Tat pathway in bacteria, such as *B. subtilis*.

MATERIALS AND METHODS

Plasmids, bacterial strains, media and growth conditions.

The plasmids and bacterial strains used in this study are listed in Table II. Strains were grown with agitation at 37ºC in either Luria Bertani (LB) medium, S7 minimal salt medium or Paris minimal (PM) medium (see [41] for exact media compositions). If appropriate, media were supplemented with antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml (*E. coli*); erythromycin (Em), 100 μg/ml (*E. coli*) or 2 μg/ml (*B. subtilis*); chloramphenicol (Cm), 5 μg/ml (*B. subtilis*); tetracycline (Tc), 10 μg/ml (*B. subtilis*); spectinomycin (Sp), 100 μg/ml (*B. subtilis*); kanamycin (Km), 50 μg/ml (*E. coli*) or 20 μg/ml (*B. subtilis*). To visualize α-amylase activity (specified by the *amyE* gene), LB plates were supplemented with 1% starch.

General DNA techniques.

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as previously described [42]. Chromosomal DNA of *B. subtilis* was isolated according to Bron and Venema [43]. PCR was carried out with the Pwo DNA polymerase, using chromosomal DNA as a template. All PCR fragments were ligated in pUC18 and subsequently introduced in *E. coli* DH5α. Plasmid DNA from *E. coli* was isolated using the alkaline lysis method [42], or the High Pure Plasmid Isolation Kit according to the protocol supplied by the manufacturer (Roche Applied Science). *B. subtilis* was transformed as described by Kunst and Rapoport [44]. All constructs were checked by sequencing.

Construction of *B. subtilis* mutant strains and plasmids.

In a recent study, we have constructed the *B. subtilis* XdsbA strain to characterize the activity of the *S. aureus* lipoprotein DsbA in *B. subtilis* [41]. This strain contains the XdsbA cassette inserted into the *amyE* locus for the xylose-inducible expression of *S. aureus dsbA*. It should be noted that the sequence coding for the mature *S. aureus* DsbA was fused to the signal sequence and ribosomal binding site of *B. subtilis mntA* to facilitate efficient translation and export of the DsbA lipoprotein. When a series of correct clones producing DsbA were checked by proteomics, one of these showed an unexpectedly high production of LipA. This serendipitously obtained LipA hyper-secreting mutant was annotated as XdsbA*.

The *B. subtilis* control strain Xtc, carrying the “empty” XTC cassette integrated in the *amyE* locus *via* double cross-over recombination was obtained by transformation of *B. subtilis* 168 with the

pXTC vector. Correct integration of the XTC cassette was checked by selection for tetracycline resistance, and screening for an AmyE-negative phenotype on starch-containing plates.

To construct plasmid pHB-ywbN-myc, the *B. subtilis ywbN* gene was PCR-amplified from chromosomal DNA of strain 168 with the primers pHB-ywbN-bsu-SalI-F (5'-GGGGGGTCGACATGTGCTATAAAAG GAG-3') and pHB-ywbN-bsu-EcoRI-R (5'-CCCCCGAATTC*TTAGTTCAAATCTTCCT CACTGATCAATTTCTG*TTCTGATTCCAG CAAACGCTGGGC-3') containing *Sal*I-*Hin*dII and *Eco*RI restriction sites, respectively (underlined). In the reverse primer a c-Myc tag sequence was incorporated (marked in italics) allowing immunodetection of the protein. The amplified *ywbN* gene was digested with *Hind*II-*Eco*RI and ligated to the *Sma*I-*Eco*RI cleaved plasmid pHB201. All plasmids thus obtained were checked by sequencing (ServiceXS; Leiden, the Netherlands). Next, the constructed pHB-ywbN-myc plasmid was introduced into the *B. subtilis* strains 168, Xtc and XdsbA*.

B. subtilis strain XdsbA* ΔTatAdCd was obtained by transformation of strain XdsbA* with chromosomal DNA of strain resistance. *B. subtilis* strain XdsbA* ΔTatAdCd_1 and selection for kanamycin resistance. *B. subtilis* strain XdsbA* ΔTatAyCy was obtained by transformation of strain XdsbA* with chromosomal DNA of strain ΔTatAyCy and selection for spectinomycin resistance. The combined mutant strain XdsbA* ΔTatAyCy ΔTatAdCd was constructed by transformation of strain XdsbA* ΔTatAyCy with chromosomal DNA of strain ΔTatAdCd_2 and selection for chloramphenicol resistance.

To construct plasmid pHB-tatAdCd, the *B. subtilis tatAd-tatCd* genes were PCR-amplified from chromosomal DNA of strain 168 with the primers TatAdCdBsF (5'-CCCCCACTAGTAAGCAATCCGATGA GGTCG-3') and TatAdCdBsR (5'-CCCCCCTCGAGGATGAGGATGTGAA GTCAC-3') containing *Spe*I and *Xho*I restriction sites, respectively (underlined). The amplified *tatAd-tatCd* genes were digested with *Spe*I-*Xho*I and ligated to the corresponding restriction sites of pHB201. Obtained plasmids were checked by sequencing. Next, the constructed pHB-tatAdCd plasmid was introduced into the *B. subtilis* strain XdsbA* ΔTatAyCy ΔTatAdCd. Finally, plasmid pCACy, carrying the *tatAy-tatCy* genes, was also introduced into the *B. subtilis* XdsbA* ΔTatAyCy ΔTatAdCd strain.

Table II. Plasmids and strains

Plasmids	Relevant properties	Reference
pUC18	ColE1; Φ80dlacZ; *lac* promoter; Ap^R	Norrander *et al.*, 1983
pXTC	Vector for the integration of genes in the *amyE* locus of *B. subtilis*; integrated genes are transcribed from the *xylA* promoter; carries the *xylR* gene; Ap^R; Tc^R	Darmon *et al.*, 2006
pXTC-dsbA	pXTC carrying *dsbA* of *S. aureus* fused to the signal sequence and RBS of *mntA* of *B. subtilis* under the transcriptional control of the *xylA* promoter; Ap^R; Tc^R	Kouwen *et al.*, 2007
pHB201	*B. subtilis*- *E. coli* expression vector; ori-pBR322; ori-pTA1060; *cat86*::*lacZa*; Cm^R; Em^R	Bron *et al.*, 1998
pHB-ywbN-myc	pHB201 vector carrying the *ywbN-myc* gene; results in the production of YwbN fused to a C-terminal Myc tag via a cysteine linker; Cm^R; Em^R	This study
pHB-tatAdCd	pHB201 vector carrying the *tatAd-tatCd* operon; Cm^R; Em^R	This study
pGDL48	*B. subtilis-E. coli* expression vector; contains multiple cloning site to place genes under the control of the erythromycin promoter; Ap^R; Km^R	Tjalsma *et al.*, 1998
pCACy	pGDL48-derivative containing the *tatAy-tatCy* operon; Ap^R; Km^R	Jongbloed *et al.*, 2004
Strains		
E. coli		
DH5α	*supE44*; *hsdR17*; *recA1*; *gyrA96*; *thi*-1; *relA1*	Hanahan, 1983
B. subtilis		
168	*trpC2*	Kunst *et al.*, 1995
Xtc	*trpC2*; *amyE*::XTC; integrated "empty" XTC cassette; Tc^R	This study
XdsbA	*trpC2*; *amyE*::XTCdsbA; integrated XTC cassette carrying *dsbA* of *S. aureus* fused to the signal sequence and RBS of *mntA* of *B. subtilis* under the transcriptional control of the *xylA* promoter; Tc^R	Kouwen *et al.*, 2007
XdsbA*	*trpC2*; *amyE*::XTC*dsbA**; XdsbA derivative with an additional unknown mutation resulting in *lipA* hyper-production; Tc^R	This study
XdsbA* ΔtatAdCd	XdsbA*; *tatAd-tatCd*::Km; Tc^R; Km^R	This study
XdsbA* ΔtatAyCy	XdsbA*; *tatAy-tatCy*::Sp; Tc^R; Sp^R	This study
XdsbA* ΔtatAyCy ΔtatAdCd	XdsbA*; *tatAy-tatCy*::Sp; *tatAd-tatCd*::Cm; Tc^R; Sp^R; Cm^R;	This study
XdsbA* ΔtatAyCy ΔtatAdCd pHB-AdCd pCaCy	XdsbA*; *tatAy-tatCy*::Sp; *tatAd-tatCd*::Cm; pHB-AdCd; pCACy; Tc^R; Sp^R; Cm^R; Em^R; Km^R;	This study
ΔtatAdCd_1	*trpC2*; *tatAd-tatCd*::Km; Km^R; previously referred to as tatAdCd	Jongbloed *et al.*, 2004
ΔtatAdCd_2	*trpC2*; *tatAd-tatCd*::Cm; Cm^R; previously referred to as ΔtatAdCd	Jongbloed *et al.*, 2002
ΔtatAyCy	*trpC2*; *tatAy-tatCy*::Sp; Sp^R	Jongbloed *et al.*, 2002

SDS-PAGE and Western blotting.

The presence of LipA, DsbA and YwbN-Myc in growth medium and/or cell lysates was detected by Western blotting. Cellular or secreted proteins were separated by SDS-PAGE (using pre-cast Bis-Tris NuPAGE gels from Invitrogen), and proteins were then semi-dry blotted (75 min at 1 mA / Cm^2) onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). Subsequently, the DsbA proteins were detected with specific polyclonal antibodies raised in rabbits (Eurogentec). LipA was detected with polyclonal antibodies kindly provided by Dr Y. L. Boersma. The YwbN-Myc protein was detected with monoclonal antibodies against the Myc-tag (Gentaur). The detection of these antibodies was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Densitometric image analysis to quantify relative protein amounts as detected by Western blotting was performed with the program ImageJ (http://rsbweb.nih.gov/ij/).

Northern blot analysis.

Preparation of total RNA was carried out as described by Eymann *et al.* (45). Northern blot analyses using specific RNA probes were performed as described by Homuth *et al.* (46). Chemiluminescence was detected using a Lumi-Imager (Roche Diagnostics). Transcript sizes were determined by comparison with an RNA size marker (Invitrogen). The digoxygenin-labelled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase and specific PCR products as templates. Synthesis of the DNA templates was performed by PCR using the following pairs of oligonucleotides: for the *lip* probe, lip-for (5'-ATGAAATTTGTAAAAAGAAG-3') and lip-rev (5'-CTAATACGACTCACTATAGGGAGAAATCAGGCTGTTGACTTGGC-3'); for *dsbA*, dsbA-for (5'-TGCGGTAAAAAAGAATCAGC-3') and dsbA-rev (5'-CTAATACGACTCACTATAGGGAGACTATTTGATTTTATCTTTTA-3'); for D1, D1-for (5'-GTAAATGCGGCAGTCAAATA-3') and D1-rev (5'-CTAATACGACTCACTATAGGGAGATGGGTCCGCCGGTGTCATTA-3'); for D2, D2-for (5'-GCAGCATGAAACCAGCTAGT-3') and D2-rev (5'-CTAATACGACTCACTATAGGGAGACTTCTGAGGGCTTGGTTTCG-3'). The underlined sequences indicate the T7 promoter region.

Transcriptome analysis.

Cell harvesting and preparation of total RNA were performed as described

previously (45). RNA samples were DNAse-treated with the RNAse-free DNAse kit (Qiagen) according to the manufacturer's instructions and purified using RNeasy mini columns (Qiagen). The quality control of the RNA preparations was performed with the RNA 6000 Nano LabChip Kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer according to the manufacturer's instructions. The RNA samples obtained from three independent cultivations were used for independent cDNA synthesis and DNA array hybridization. Generation of the Cy3/Cy5-labeled cDNAs and hybridization to *B. subtilis* whole-genome DNA microarrays (Eurogentec) were performed as previously described by Jürgen *et al*. (47). The slides were scanned with a ScanArray Express scanner (PerkinElmer Life and Analytical Sciences). Quantification of the signal and background intensities of individual spots was carried out using the ScanArray Express image analysis software.

Data were analyzed using the GeneSpring software (Agilent Technologies). Raw signal intensities were first transformed by intensity dependent LOWESS normalization. The normalized array data were subjected to a statistical analysis using Cyber-T, a program based on a *t*-test combined with a Bayesian statistical framework (48). The software is accessible through a Web interface at http://cybert.microarray.ics.uci.edu. The mRNA abundance was considered to be significantly different between the wild type and the respective mutant strain if (i) the Cyber-T Bayesian *P* value was < 0.001 and (ii) the individual fold change was at least 2. The potential and known functions of the encoded proteins were predominantly inferred from the SubtiList database (http://genolist.pasteur.fr/SubtiList/).

Proteomics.

Cells of *B. subtilis* were grown at 37°C under vigorous agitation in 1 liter of LB medium, or a synthetic medium containing 0.16 mM KH_2PO_4 to induce a phosphate starvation response (49). At the onset of and after 1 hour of post-exponential growth, cells were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were collected for two-dimensional gel electrophoresis (2D PAGE), gels were stained with the SYPRO Ruby protein gel stain (Molecular Probes Inc.). All detected protein spots were identified previously by matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS) (7, 9, 34, 39). To visualize possible differences in extracellular protein composition, dual channel image analysis of stained gels was performed using the DECODON Delta 2D software (http://www.decodon.com). Each experiment was performed at least twice.

Pulse labeling of proteins, immunoprecipitation, and fluorography. Pulse labeling of *B. subtilis*, immunoprecipitation, and fluorography were performed as described previously (39, 50). To inhibit the translocation ATPase activity of SecA, sodium azide (1.5 mM final concentration) was added to the cells 5 min prior to labeling (51). Immunoprecipitation was performed with specific antibodies against LipA.

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CONFLICTS OF INTEREST

The authors declare that they have no financial/commercial conflicts of interest.

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Chapter 4

Environmental salinity determines the specificity and biological function of Tat-dependent protein secretion by *Bacillus subtilis*

René van der Ploeg, Ulrike Mäder, Georg Homuth, Marcus Miethke, Mohamed A. Marahiel, Colin R. Harwood, Michael Hecker, Haike Antelmann, and Jan Maarten van Dijl

ABSTRACT

Twin-arginine protein translocation (Tat) pathways are required for transport of folded proteins across bacterial, archaeal and chloroplast membranes. Recent studies indicate that Tat has evolved into a mainstream pathway for protein secretion in halophilic archaea, which thrive in highly saline milieus. Here, we investigated the effects of environmental salinity on Tat-dependent protein secretion by the Gram-positive soil bacterium *Bacillus subtilis*, which can encounter widely differing salt concentrations in its natural habitat. The results show that environmental salinity determines specificity and usage of the *B. subtilis* Tat pathway. At high salinity, the two Tat translocases of *B. subtilis*, TatAdCd and TatAyCy, cooperate in the secretion of the Dyp-type peroxidase YwbN and the protease WprA. By contrast, YwbN is exclusively secreted via the TatAyCy translocase at low salinity, and the same seems true for the cell wall hydrolase LytD. At low salinity, *tatAyCy* or *ywbN* mutants display significantly reduced exponential growth rates and severe cell lysis. This is due to a critical role of secreted YwbN in the acquisition of iron under these conditions. Taken together, our findings show that environmental conditions, such as salinity, can determine the specificity, substrate spectrum and biological function of Tat-dependent protein secretion in a bacterium.

Submitted for publication

INTRODUCTION

The transport of proteins across biological membranes and their subsequent secretion into external milieus are vital processes for all known microorganisms. These processes depend on the activity of dedicated molecular machines. A first critical step in protein secretion is the passage of transported proteins through the cytoplasmic membrane, which can occur either in an unfolded state *via* the general secretion (Sec) machinery, or in a folded state *via* the twin-arginine translocation (Tat) machinery [1-5]. Accordingly, the Sec and Tat machines work independently of each other, using distinct mechanisms for protein translocation. Acceptance of a protein by the Sec or Tat complexes is dictated by the presence of an N-terminal signal peptide with or without a selective recognition motif for Tat (*i.e.* the twin-arginine motif) [6], and the folding state of the transported protein [7-9]. In general, Tat-dependent proteins fold prior to translocation, while the folding of Sec-dependent proteins occurs post-translocationally [7,8].

Early studies have defined the twin-arginine (RR-) motif in the N-region of signal peptides as S/T-R-R-x-F-L-K [6,10-13]. However, this motif is not always strictly conserved in Tat-dependently exported proteins. The observed natural variations, as well as site-directed mutagenesis studies, have defined a more general RR-consensus sequence as R/K-R-x-#-#, where # is a hydrophobic residue [14-16]. This RR-motif is recognized by the membrane-embedded Tat machinery of which two general types are known: Gram-negative bacteria, like *Escherichia coli*, contain a TatABC-type machinery consisting of three Tat proteins (TatA, TatB and TatC) that are indispensable for translocation [17,18], while most Gram-positive bacteria contain a "minimal" TatAC machinery that lacks the TatB protein [19,20]. In these TatAC translocases, the role of TatB is fulfilled by a bifunctional TatA protein [21,22]. The precise mechanism of Tat-dependent protein translocation is currently unknown, but studies in *E. coli* and thylakoids have shown that RR-signal peptide recognition involves a TatB-TatC complex [23-25]. Subsequently, TatBC-precursor complexes merge with TatA sub-complexes to facilitate the translocation process in such a way that folded proteins of varying sizes and complexity can pass the membrane [25,26].

Notably, some organisms contain multiple Tat translocases [27]. This was shown for the Gram-positive bacterium *Bacillus subtilis*, which contains two TatAC translocases named TatAdCd and TatAyCy [21]. The genes coding for these translocases are organised in operons at independent genomic loci [19]. Furthermore, *B. subtilis* contains a third

tatA gene (*tatAc*), the function of which is currently unknown [20,21]. The specificities of the TatAdCd and TatAyCy complexes are non-identical, but overlapping. Only two *B. subtilis* proteins, YwbN and PhoD, are secreted strictly in a Tat-dependent manner [20,21]. YwbN is a member of the family of Dyp-type peroxidases that contain a heme co-factor [28]. This protein is a preferred substrate for the constitutively expressed TatAyCy translocase. In contrast, secretion of the phosphodiesterase PhoD is strictly dependent on the TatAdCd translocase. Notably, the *phoD* gene is located upstream of the *tatAd-tatCd* genes, and all three genes are induced under phosphate starvation conditions [19].

In silico predictions based on the detection of potential RR-motifs in signal peptides have suggested that there are 69 potentially Tat-dependently secreted proteins in *B. subtilis* [20]. However, proteomics and molecular biological analyses revealed that only two of these - YwbN and PhoD – are strictly dependent on a functional Tat translocase. The signal peptides of the *B. subtilis* QcrA and YkuE proteins were shown to direct Tat-dependent protein secretion in Streptomyces [29], but the Tat-dependence of these proteins in *B. subtilis* remains to be shown. Very recently, we demonstrated that the esterase LipA can be secreted via Sec and Tat in *B. subtilis* [30]. This became evident under conditions of LipA hyperproduction, indicating that an overflow mechanism exists to re-direct LipA from the normally used Sec-dependent export route into the Tat-dependent route. This suggests that seemingly Sec-dependent proteins may turn into Tat-dependent proteins depending on intracellular and perhaps even extracellular conditions. In this context, it is noteworthy that the Tat machinery is extensively used for protein secretion by halophilic archaea, such as *Haloferax volcanii* and *Haloarcula hispanica*, which supports the idea that Tat has evolved into a mainstream secretion pathway for organisms that grow in highly saline milieus [31-36]. This raised the question as to what extent salinity can impact on Tat-dependent protein secretion in microorganisms that live in ecological niches where the salinity can fluctuate markedly. To answer this question, we studied the influence of salt on protein secretion by the soil bacterium *B. subtilis.* The results show that the NaCl content of the growth medium has a very strong impact on Tat-dependent protein transport. Remarkably, the specificity of the two Tat translocases for the YwbN protein changed when cells were grown at high salinity and, under these conditions, the WprA quality control protease was identified as a novel Tat-dependent exoprotein of *Bacillus*. At low salinity, the TatAyCy translocase was shown to be of major importance for

growth and cell viability, revealing an essential role of YwbN in iron acquisition. In addition, the cell wall hydrolase LytD was identified as a novel potential Tat substrate in *Bacillus*.

RESULTS

TatAyCy-independent secretion of YwbN at high salinity.

As a first approach to investigate whether high salinity might impact on Tat-dependent protein secretion, we investigated the secretion of YwbN in LB medium with 6% salt. To facilitate YwbN detection, this protein was provided with a C-terminal Myc-epitope, and the corresponding gene construct was ectopically expressed from a xylose-inducible promoter. When the cells reached an OD_{600} of 2, expression of *ywbN* was induced and growth was continued for 3 hours. Cellular and growth medium fractions were collected and used for SDS-PAGE and Western blotting to monitor YwbN secretion (Fig. 1, upper panels). The results revealed a very strong impact of high salinity on the specificity of YwbN secretion. Clearly, YwbN secretion was no longer strictly TatAyCy-dependent when cells were grown in medium with 6% salt; although deletion of *tatAyCy* resulted in a strong reduction of the extracellular level of YwbN, the *tatAyCy* mutant nevertheless secreted substantial amounts of this protein. This contrasts markedly with the secretion of YwbN by cells grown at 1% salt, which is strictly TatAyCy-dependent (Fig. 1, compare upper and middle panels). Perhaps even more striking, at 6% NaCl the secretion of YwbN was also strongly reduced in a *tatAdCd* mutant, showing for the first time an involvement of the TatAdCd translocase in YwbN secretion. These findings imply that the TatAyCy and TatAdCd translocases have a cooperative mode of action under conditions of high salinity. In fact, comparable levels of mature YwbN were secreted by the *tatAyCy* mutant, the *tatAdCd* mutant and the total-*tat* mutant that lacks all five *B. subtilis tat* genes. These data show that YwbN secretion was partially Tat-independent when cells were grown in the presence of 6% salt. To verify that this Tat-independent secretion of YwbN was not due to cell lysis, the locations of the secreted protein LipA and the cytoplasmic marker protein TrxA and were verified by Western blotting. No extracellular TrxA was detected, indicating that the observed Tat-independent secretion of YwbN was not due to cell lysis (not shown). Furthermore, secreted LipA was readily detectable at 6% salt (Fig. 1), and Sypro Ruby-staining of gels loaded with growth medium samples did not reveal any

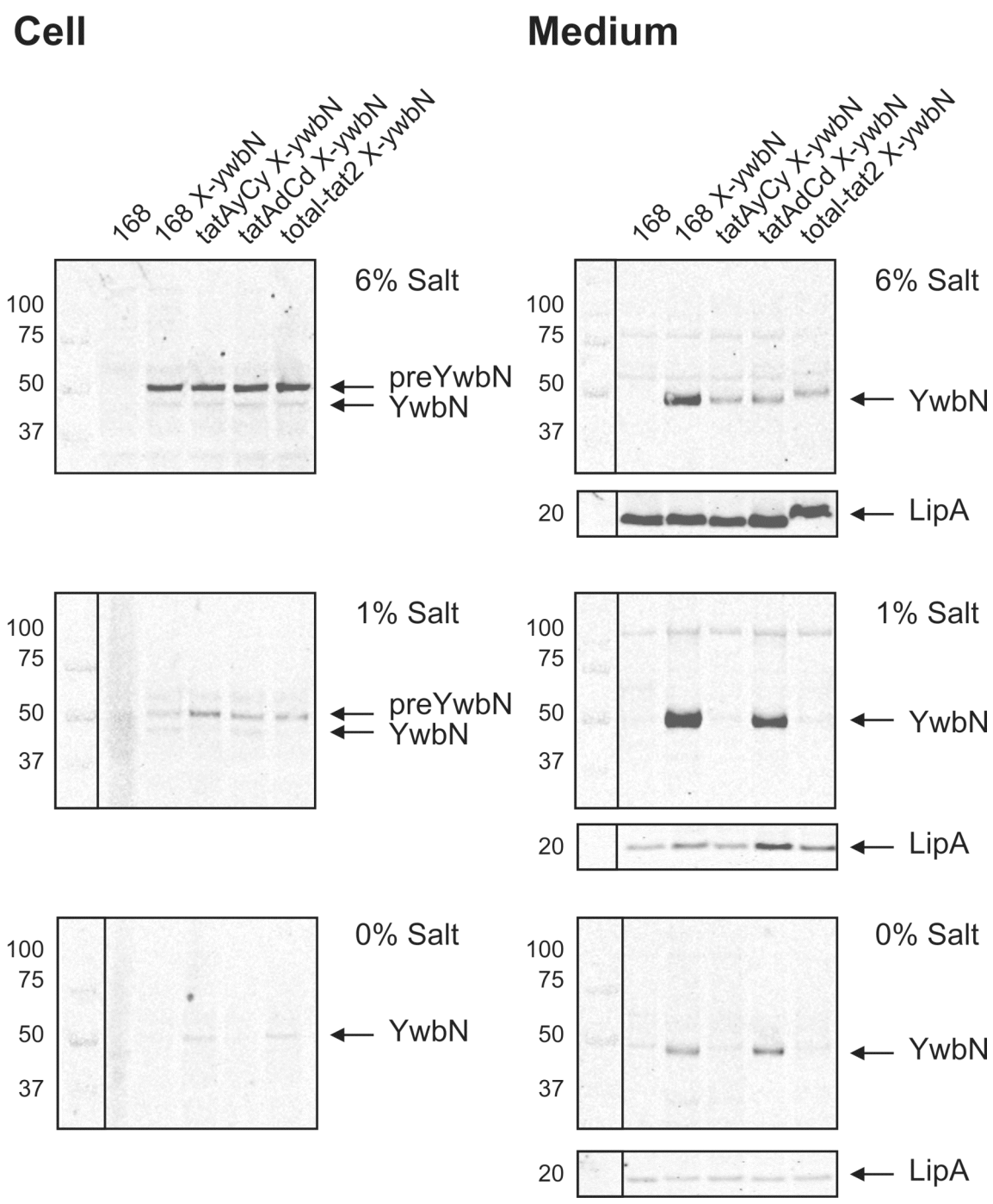


Figure 1. Tat-dependence of YwbN secretion in media with differing salinity. Cell and medium fractions of *B. subtilis tat* mutant strains and the parental strain 168 were separated and used for SDS-PAGE and Western blotting using specific antibodies. From top to bottom the panels show results obtained for cells grown in LB with 6%, 1% or 0% added NaCl. Protein loading was corrected for OD_{600}. The YwbN-Myc and LipA proteins, and Mw markers are indicated.

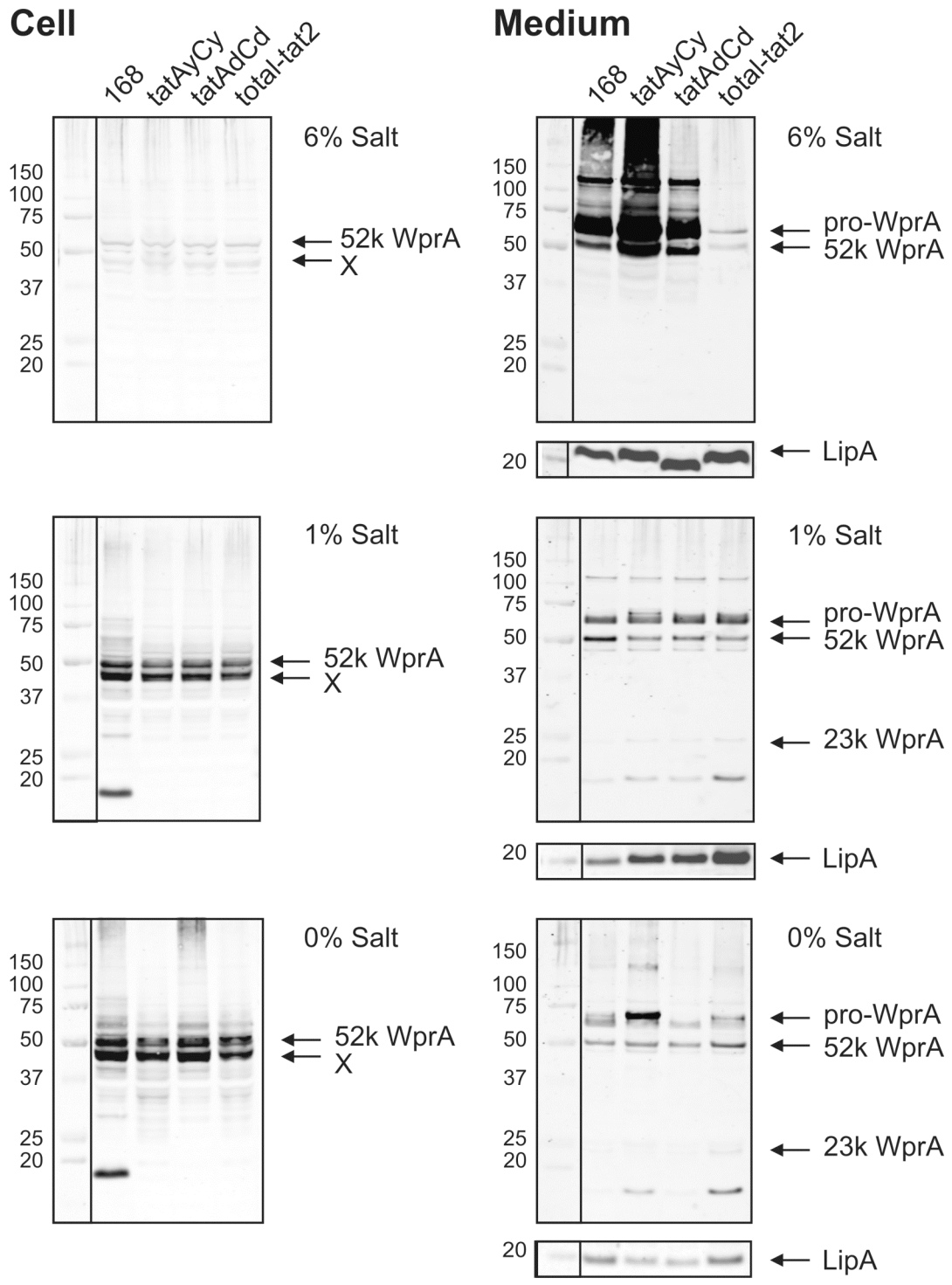


Figure 2. Tat-dependence of WprA secretion in media with differing salinity. Cell and medium fractions of *B. subtilis tat* mutant strains and the parental strain 168 were separated and used for SDS-PAGE and Western blotting using polyclonal antibodies specific for 52k WprA. From top to bottom the panels show results obtained for cells grown in LB with 6%, 1% or 0% added NaCl. Protein loading was corrected for OD_{600}. WprA-specific processing products, LipA and Mw markers are indicated. X indicates a ~45 kDa cell-associated WprA product. Pro-WprA indicates a precursor form of WprA that probably contains the 52-kDa fragment *plus* the 17-kDa linker region.

differences in the total amounts of protein secreted by the different strains (not shown). Taken together, these findings show that the TatAyCy and TatAdCd translocases of *B. subtilis* have altered preferences for YwbN under conditions of high salinity and that both translocases cooperate under these conditions.

The quality control protease WprA is secreted TatAyCy-dependently at high salinity.

To investigate whether high salinity might impact on the secretion of proteins other than YwbN, we used a proteomics approach to compare the secretomes of the total-*tat* mutant and the parental 168 strain in LB medium with 6% salt. The results indicated that the cell wall-associated and extracellular serine protease WprA might be secreted Tat-dependently at high salinity (not shown). Interestingly, we had previously shown that WprA is secreted Tat-independently in standard LB medium with 1% salt [20]. Therefore, we compared the secretion of WprA by *tatAyCy*, *tatAdCd* and total-*tat* mutant strains at 6% and 1% salt. In this respect it is relevant to note that the 96-kDa WprA is post-translocationally processed into at least two major products of 52 and 23 kDa, the 52-kDa fragment representing the WprA domain with protease activity [37-39]. Cultures were grown to an OD_{600} of 2, and the presence of processed WprA products in the cell and growth medium fractions was determined by Western blotting and immune detection with polyclonal antibodies raised against the 52 kDa product of WprA. The results show that, compared to standard LB medium with 1% salt, the amounts of extracellular WprA products (52 kDa and a pro-form of ~60 kDa) were drastically increased in medium with 6% salt, while the cellular levels of these products were reduced (Fig. 2, upper panels). In LB medium without added salt and LB medium with 1% salt, no Tat-dependent secretion of WprA was observed, although some minor differences in WprA processing were detectable in the different *tat* mutant strains. In contrast, the secretion of WprA products was strongly Tat-dependent when cells were grown in medium with 6% salt. Notably, under these conditions of high salinity both TatAdCd and TatAyCy were required for high-level secretion of WprA products, which confirms the active role of the TatAdCd translocase at high salinity as observed in the secretion of YwbN (Fig. 1). Furthermore, deletion of *tatAdCd* by itself had no influence on WprA secretion, whereas deletion of *tatAyCy* resulted in elevated levels of WprA secretion. The latter finding is fully in line with previous observations for two other Tat substrates of *B. subtilis*, namely PhoD [19] and hyperproduced LipA [30], whose secretion was enhanced in the absence of a functional TatAyCy translocase, but completely

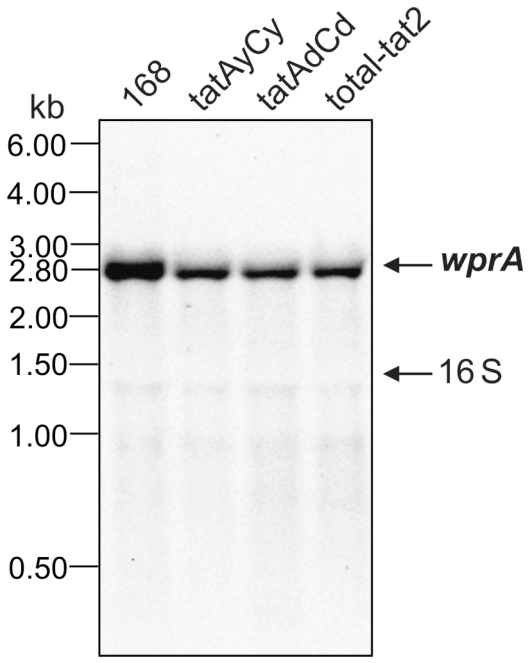


Figure 3. Transcription of *wprA* in *tat* mutant strains grown at high salinity. *Tat* mutant strains and the parental strain 168 were grown in LB medium with 6% NaCl as in Figure 2. Cells were harvested at an OD_{600} of 2. Expression of *wprA* was assessed by Northern blotting (5 µg per lane).

blocked in the absence of functional TatAdCd and TatAyCy translocases (note that LipA was not hyperproduced in the present experiments and that secretion of LipA as in Figs. 1 and 2 is thus Sec-dependent). These findings indicate some level of interference between the two Tat translocases of *B. subtilis*. To rule out any indirect effects of relevant *tat* mutations on the expression of *wprA* at growth under conditions of high salinity, we performed a Northern blotting analysis (Fig. 3). The results showed that the *wprA* transcript ratios for the *tatAyCy*, *tatAdCd* and total-*tat*-mutant strains compared to the parental strain 168 were on average ~0.5. These relatively minor differences in amounts of *wprA* transcript between the *tat* mutants and the 168 strain cannot account for the vastly different secretion levels as shown in Figure 2. Taken together, these findings show that the secretion of the WprA products under conditions of high salinity is directly related to the presence of active TatAdCd and TatAyCy translocases, which supports the view that WprA is a substrate for these translocases depending on the growth conditions. If so, the non-translocated WprA in total-*tat* mutant cells grown at high salinity is probably degraded.

TatAyCy is required for optimal growth of *B. subtilis* and entry into stationary phase at low salinity.

To investigate whether conditions of low salinity would also impact on Tat-dependent protein secretion, the *tatAdCd*, *tatAyCy* and total-*tat* mutant strains were cultivated in LB medium containing no added salt. Growth of these strains at 37 ºC was followed for 8 hours, which revealed an unexpectedly strong growth phenotype for mutant strains lacking the *tatAy* and/or *tatCy* genes (Fig. 5A). Compared to the parental strain 168 and *tatAdCd* mutant strains, the *tatAyCy* and total-*tat* mutant strains showed significantly reduced growth rates during the exponential growth phase. Most strikingly, the *tatAyCy* and total-*tat*

mutants exhibited a lower growth yield as compared to the *tatAyCy* proficient strains and they were even unable to enter directly into a stationary phase of growth. Instead, mutants lacking *tatAyCy* displayed a severe lysis phenotype as reflected by a significant drop in the OD_{600}. Interestingly, the surviving *tatAyCy* mutant cells resumed (or continued) growth about 1.5 hours after cell lysis started to occur, but at a lower rate than during the initial exponential growth. This growth phenotype could be reversed by complementation of the chromosomal *tatAyCy* mutations with plasmid-borne copies of the *tatAyCy* genes (Fig. 5B). Specifically, the growth defect of cells lacking *tatAy* and *tatCy* at low salinity was only reversed when both the *tatAy* and *tatCy* genes were present on the complementing plasmid. Since *tatAyCy* mutant cells that had recovered from the lysis phase showed the same phenotype upon re-cultivation under low salinity conditions, it seems that the cells that resumed growth had only adapted to these conditions. Importantly, the observed phenotype revealed a clear link between environmental salinity and the requirement for an active Tat system.

Secretion of the autolysin LytD is TatAyCy-dependent at low salinity.

To test whether the observed low-salinity lysis phenotype of strains lacking *tatAyCy* might relate to autolysin production or secretion, we performed zymogram assays with exponentially growing cells and their growth medium fractions. This allows detection of the activities of two major *B. subtilis* autolysins, LytC and LytD, which account for ~95% of the autolysin activity during vegetative growth [40]. Interestingly, at low salinity, both strains lacking *tatAyCy* (*i.e.* the *tatAyCy* and total-*tat* mutants) did not secrete LytD into the growth medium, while the LytD secretion

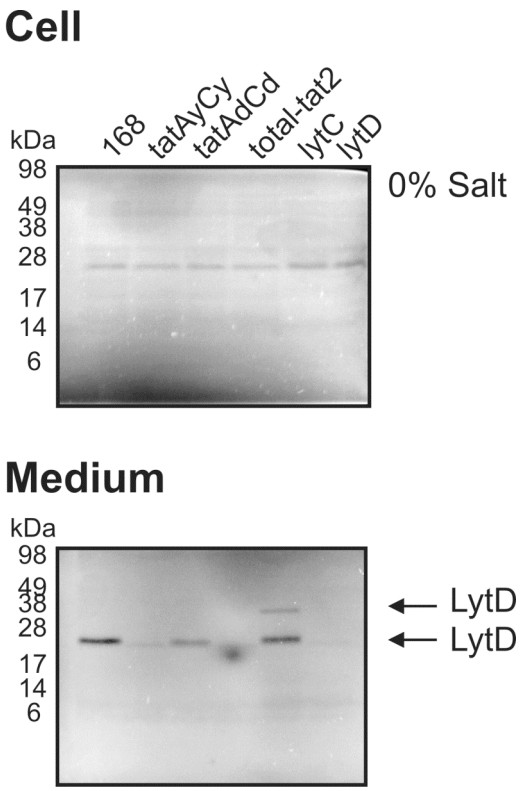
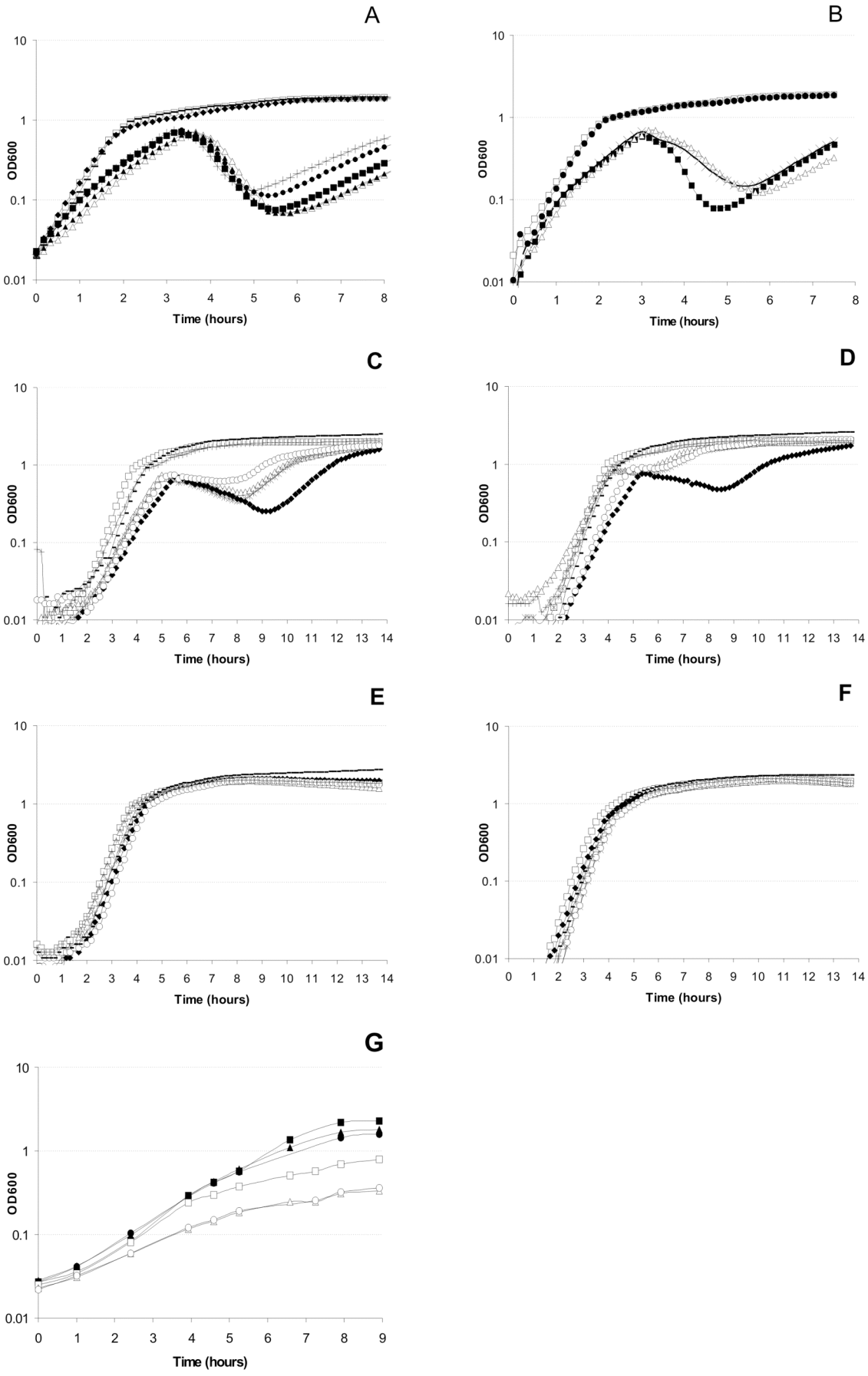


Figure 4. Tat-dependence of LytD secretion at low salinity. Cells of *B. subtilis tatAyCy*, *tatAdCd*, total-*tat2*, *lytC* or *lytD* mutant strains were grown in LB medium without added NaCl. Activity of autolysins in cellular fractions (upper panel) and growth medium fractions (lower panel) was detected by zymography. Positions of Mw marker proteins are indicated.

was not affected in the *tatAdCd* mutant (Fig. 4). This shows that LytD secretion depends specifically on TatAyCy under the tested conditions. Notably, the TatAyCy dependence of LytD secretion was specific for conditions of low salinity as it was not observed in media with 1% or 6% salt (data not shown). Furthermore, the *tatAyCy* mutations had no detectable effect on the cellular autolysin levels, suggesting that the observed lysis phenotype at low salinity did not relate to autolysin production. This strains lacking *tatAyCy* in the absence of added salt (Fig. 5C), while its growth phenotype was normal in the presence of 1% salt (Fig. 5F). Furthermore, the growth phenotype of the *ywbN* mutant strain was fully complemented by xylose-induced ectopic expression of a copy of *ywbN-Myc* that was integrated into the chromosomal *amyE* locus (Fig. 5C). Consistent with these findings, the secretion of Myc-tagged YwbN was strictly dependent on TatAyCy under conditions of low salinity (Fig. 1, lower panels). Since *ywbN* is part of a well-conserved cluster of three genes of which the two other genes, *ywbL* and *ywbM*, have been implicated in iron uptake [21,41], we also investigated whether *ywbL* or *ywbM* mutant strains have a growth defect at low salinity. Indeed, this was the case (Fig. 5C), suggesting that YwbL and YwbM, as well as TatAyCy-dependently secreted YwbN could be required for the uptake of sufficient amounts of iron to sustain growth view was confirmed by the observation that neither *lytC* nor *lytD* mutant strains showed growth defects at low salinity (not shown).

TatAyCy-dependent secretion of YwbN has an important role in iron acquisition. To test whether the known TatAyCy substrate YwbN was implicated in the growth kinetics observed at low salinity, experiments were performed with a *ywbN* mutant. Indeed, the *ywbN* mutant strain displayed a similar growth phenotype as under conditions of low salinity. To test this idea, all growth experiments at low salinity were repeated in the presence of 10 μM $FeCl_3$ (Fig. 5D) or $FeSO_4$ (Fig. 5E). The

Figure 5. Growth phenotypes of *tatAyCy* and *ywbLMN* mutant strains at low salinity. *B. subtilis tat* mutant strains or the parental strain 168 were grown for 8 to 14 hours in LB medium (**A-F**), or BOC medium (**G**). **A**, **B**, **C**, **D**, and **E**, LB medium without NaCl. For some experiments the LB medium was supplemented with 10 μM $FeCl_3$ (**D**), 10 μM $FeSO_4$ (**E**), or 1% NaCl (**F**). **A.** Growth of *tat* mutant strains: *tatAd tatAy* (+), *tatCd* (filled diamonds), *tatCy* (filled triangles), *tatCd tatCy* (filled circles), *tatAdCd* (filled rectangles), *tatAyCy* (open triangles), total-*tat*2 (filled squares). Parental strain 168 (open squares). **B.** Growth of the *tatAyCy* mutant strain complemented with *tatAy* (pCAy; open triangles), *tatCy* (pCCy; X), or *tatAyCy* (pCACy; filled circles). Controls: *tatAyCy* mutant with empty vector pGDL48 (closed squares), parental strain 168 (open squares). **C-F.** Growth of mutant strains: *tatAyCy* (open triangles), *ywbL* (closed diamonds), *ywbM* (X), *ywbN* (open circles), *ywbN* X*ywbN* (no xylose; +), *ywbN* X*ywbN* (plus xylose; closed rectangles). Control: parental strain 168 (open squares). **G.** Growth of strains in BOC medium: total-*tat* (triangles), total-*tat2* (circles) and 168 (squares). Open symbols, no added iron; closed symbols, addition of 10 μM $FeSO_4$.


A
B
C
D
E
F
G
OD600
Time (hours)

results show that addition of 10 μM Fe^{2+} to the low salinity LB medium strongly stimulated the growth of the *tatAyCy*, *ywbL*, *ywbM*, or *ywbN* mutants to levels that were indistinguishable from growth in the presence of 1% salt (Fig. 5, compare panels E and F). By contrast, the addition of 10 μM Fe^{3+} to the low salinity medium had only a moderately stimulating effect on the growth of the *tatAyCy*, *ywbM* or *ywbN* mutant strains, while Fe^{3+} had barely any growth-promoting effect on the *ywbL* mutant strain (Fig. 5D). Limited rescue of the mutants by addition of Fe^{3+} is consistent with the lack of Fe^{3+}-specific bacillibactin-dependent iron acquisition in the 168 background [42], while excess of Fe^{2+} is directed towards low affinity uptake by divalent cation importers [43,44]. The results imply that YwbL is the decisive Fe^{3+} permease for elemental iron uptake, and that the *B. subtilis* TatAyCy translocase and its substrate YwbN are of pivotal importance for the acquisition of iron during growth at low salinity. Since this suggests a possible general role for the *B. subtilis* Tat pathway in growth under iron-limited conditions, we tested the growth of two independently constructed *B. subtilis* total-*tat* mutants in synthetic iron-limited BOC medium. The results in Figure 5G show that, indeed, the growth rates of total-*tat* mutants in BOC medium were significantly lower than that of the parental strain 168. This growth defect was largely relieved by the addition of 10 μM Fe^{2+} to the medium (Fig. 5G), showing that the *B. subtilis* Tat pathway is required for optimal growth under iron-limited conditions.

DISCUSSION

In the present studies we have addressed the question as to whether environmental salinity is a determinant in Tat-dependent protein secretion by *B. subtilis*. The results show that, indeed, changes in environmental salinity do have a major impact on the specificity and usage of the *Bacillus* Tat pathway for protein secretion. Experiments performed at high salinity growth conditions revealed clear changes in the specificity of the Tat pathway for the known Tat substrate protein YwbN and they allowed identification of the quality control protease WprA as a salinity-dependent Tat substrate. Conversely, experiments with cells grown at low salinity revealed Tat-dependent secretion of the cell wall hydrolase LytD. Importantly, the studies showed that the TatAyCy translocase and its substrate YwbN have an essential role in iron acquisition at low salinity.

Intriguingly, high salinity had a strong impact on the specificity of the Tat pathway

of *B. subtilis*, as shown by the role of the TatAdCd translocase in YwbN secretion. The obervation that, at high salinity, TatAdCd was involved in YwbN secretion to the same extent as TatAyCy implies that the Tat translocases of *B. subtilis* cooperate under these conditions. This contrasts strongly with the previously demonstrated exclusive dependence of YwbN secretion on the TatAyCy translocase, when cells were grown in LB with 1% salt [21]. It was recently reported that YwbN can be secreted in a TatAdCd dependent manner by cells growing in LB medium with 1% NaCl. However, this only occurs when the TatAdCd translocase is overproduced artificially [45]. The overproduced TatAdCd was able to sustain YwbN secretion in the absence of TatAyCy, which is clearly not the case at high salinity conditions where cooperation between the TatAdCd and TatAyCy translocases appears to be a strict requirement for YwbN secretion. At present, it is not clear whether the observed cooperativity between the two Tat translocases is triggered directly by high levels of salt.

Cells grown at high salinity secreted substantial amounts of YwbN in a Tat-independent manner. This may relate to salt-induced specificity changes in the Tat translocases of *B. subtilis* but, knowing that the Tat system has probably evolved to transport folded proteins, it seems more likely that the Tat-independent secretion of YwbN reflects an influence of the high salt conditions on the folding of YwbN. For example, if high salinity reduces the rate of folding of YwbN this might increase its compatibility with the Sec pathway. Alternatively, since the *E. coli* YwbN-homolog EfeB (YcdB) was shown to carry an iron-heme cofactor assembled into the protein before transport [28], the previously documented high salinity-induced iron limitation in *B. subtilis* [46,47] may impact on cytoplasmic cofactor assembly and thus impair Tat-dependent translocation of the *holo*-YwbN protein. Conversely, an enhanced rate of folding might be the reason why the WprA quality control protease was identified as a Tat-dependently secreted protein at high salinity, but not at low salinity conditions. An increase in the rate of cytoplasmic folding at high salinity would reduce the Sec-dependent secretion of WprA, which in turn would allow recognition of the potential RR-motif RRKFSS in the WprA signal peptide by Tat. Like YwbN, this seems to involve the cooperative action of the TatAdCd and TatAyCy translocases. The finding that the serine protease WprA can be transported Tat-dependently under particular conditions is reminiscent of our previous observation that the related serine protease subtilisin (AprE) of *B. subtilis* can be secreted Tat-dependently when provided with the RR-signal peptide of YwbN [48]. This indicates that serine proteases like

subtilisin and WprA have an intrinsic potential for Tat-dependent secretion.

At low salinity growth conditions, the cell wall hydrolase LytD was shown to be secreted TatAyCy-dependently, which is in line with the presence of the potential RR-motif KRLIAP in the signal peptide of this protein. The export of LytD is known to be required for cell separation during vegetative growth and motility during stationary phase [49], but a potential Tat-dependence of this protein was so far not noticed. As suggested above for YwbN and WprA, this Tat-dependence may relate to influences of environmental salinity on the folding of LytD prior to export from the cytoplasm. If so, this would imply that low salinity slows down LytD folding thereby making the protein compatible with Tat-dependent transport. However, due to the altered growth rates of *B. subtilis tatAyCy* mutant cells at low salinity, we cannot rule out the possibility that the observed effects of *tatAyCy* mutations on LytD secretion are indirectly related to the absence of the TatAyCy translocase.

The present experiments revealed an essential role of the TatAyCy secreted YwbN in the acquisition of iron during growth in LB at low salinity and in iron-limiting synthetic BOC medium. At low salinity, the growth defects and post-exponential growth lysis phenomenon of *tatAyCy* or *ywbN* mutants could be fully suppressed by the addition of Fe^{2+}, and to a lesser extent Fe^{3+}, to the growth medium. This implies that cells lacking TatAyCy and/or YwbN are iron-starved at low salinity. The same turned out to be true for cells lacking the lipoprotein YwbM and the integral membrane protein YwbL. Interestingly, the *E. coli* homologue of YwbM, named EfeO, is a periplasmic protein that has been implicated in high-affinity iron-binding [41]. YwbL is a homologue of the EfeU iron permease in the *E. coli* inner membrane [41,50], and the high-affinity iron permease Ftr1p of fungi and yeast [51]. While Ftr1p is an Fe^{3+} permease [51,52], EfeU was shown to permeate Fe^{2+} *in vitro* into proteoliposomes [50]. The present study shows impaired growth of a *ywbL* mutant in the presence of Fe^{3+}, but not in the presence of Fe^{2+}, indicating that *B. subtilis* YwbL is specific for Fe^{3+} uptake. Taken together, our findings show that severe iron limitation is the reason why cells lacking YwbLMN or TatAyCy grow at reduced rates in low salinity LB medium, and start to lyse instead of entering the stationary growth phase. The finding that such mutants resume growth after the lysis phase suggests that cell lysis results in the liberation of iron, which can be reused by the surviving cells. The precise role of YwbN in making iron available for the cells is not entirely clear but, being a homologue of the Dyp-type iron-dependent peroxidase EfeB, it seems most likely that this enzyme

is involved in oxidation of Fe^{2+} for subsequent uptake of Fe^{3+} by YwbL, which is in analogy to the yeast Fet3p-Ftr1p high-affinity iron uptake system [28,41]. A function of YwbN in iron metabolism in *B. subtilis* is fully consistent with the finding by Helmann and co-workers that the *ywbN* gene is part of the Fur regulon [53]. By contrast, *tatAyCy* does not seem to be controlled by Fur, which is consistent with a "mainstream" role in Tat-dependent protein translocation, as documented in the present studies. It is remarkable to note that, while an iron starvation response was previously documented for *B. subtilis* cells grown at high salinity [46,47], the apparent iron limitation under these conditions did not result in a requirement for the TatAyCy translocase as observed under conditions of low salinity.

Taken together, our present results show for the first time that environmental salinity is a critical determinant for Tat-dependent protein secretion in *B. subtilis.* Depending on the salinity levels, proteins can be directed from the Sec into the Tat pathway (*e.g.* WprA or LytD) or *vice versa* (*e.g.* YwbN). These observations are consistent with our previous observation that the esterase LipA of *B. subtilis* can be redirected from the Sec to the Tat pathway under conditions of hyperproduction *via* an overflow mechanism [30]. This suggests that there may be additional, as yet unidentified factors that can impact on the choice between Sec or Tat pathway usage. This opens up the possibility that *B. subtilis* proteins with potential RR-signal peptides, other than the ones identified here, can be secreted in a Tat-dependent manner. A parameter that can impact strongly on rates of protein (un)folding is temperature. Nevertheless, growth temperature by itself does not seem to be a factor determining secretion pathway dependency, since we did not detect any obvious differences in the Tat-(in)dependence of protein secretion when cells were grown at 15, 30, 37 or 48 ºC (unpublished observations). However, being an organism that lives in the soil, *B. subtilis* can be exposed to a plethora of different combinations of environmental insults that may impact on the pre-translocational folding of secretory precursor proteins. We are therefore convinced that more proteins will use the Sec and/or Tat pathways of *B. subtilis* in a condition-dependent manner. Identification of such conditions will be of interest not only from a fundamental scientific point of view, but also for the biotechnological application of *B. subtilis* as a cell factory for the production of high-value proteins.

MATERIALS AND METHODS

Plasmids, bacterial strains, media and growth conditions.

The plasmids and bacterial strains used in this study are listed in Table I. Strains were grown with agitation at 37ºC in Luria Bertani-Miller (LB) medium consisting of 1% tryptone, 0.5% yeast extract and 0%, 1% or 6% added NaCl, pH 7.4. Belitsky minimal medium without citrate (BOC medium) was prepared as described [54]. If appropriate, media for *B. subtilis* were supplemented with the following antibiotics: erythromycin (Em), 2 µg/ml; chloramphenicol (Cm), 5 µg/ml; tetracycline (Tc), 10 µg/ml; spectinomycin (Sp), 100 µg/ml; kanamycin (Km), 20 µg/ml.

Growth experiments.

Strains were pre-cultured in LB medium containing 1% NaCl and subsequently diluted in LB medium with 0%, 1% or 6% NaCl to an optical density at 600 nm (OD_{600}) of ~0.01. Growth was continued in triplicate wells of a 96-well black optical bottom microtiter plate (Nunc) that was incubated in a Biotek Synergy 2 plate reader (37°C, variable shaking). OD_{600} readings were recorded for 8 or 14 hours.

SDS-PAGE, Western blotting, Zymography and Proteomics.

Cellular or secreted proteins were separated by SDS-PAGE using pre-cast Bis-Tris NuPAGE gels (Invitrogen). Separated proteins were stained with SYPRO Ruby protein gel stain (Molecular Probes Inc.). The presence of LipA, TrxA, WprA or YwbN-Myc in cellular or growth medium fractions was detected by Western blotting. For this purpose, proteins separated by SDS-PAGE were semi-dry blotted (75 min at 1 mA / Cm^2) onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). Subsequently, the LipA, TrxA and WprA proteins were detected with specific polyclonal antibodies raised in rabbits. YwbN-Myc was detected with monoclonal antibodies against the Myc-tag (Gentaur). Visualisation of bound antibodies was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm. To analyze the autolysin activity in cellular and growth medium fractions, zymography was performed. For this purpose, 12.5% bisacrylamide gels containing 0.1% Micrococcal cell wall (Sigma-Aldrich) were prepared. Cell and growth medium fractions were used for SDS-PAGE, but in this particular case a non-reducing gel loading buffer was used. Prior to gel loading, the samples were incubated for 10 minutes at 95 ºC. After electrophoresis, the proteins were allowed to renature in 25 mM

Table I. Strains and Plasmids

Plasmids	Relevant properties	Reference
pGDL48	Contains multiple cloning site to place genes under the control of the erythromycin promoter; 6.8 kb; Ap^R; Km^R	Tjalsma *et al*., 1998
pCAy	pGDL48-derivative containing the *tatAy* gene; 7.0 kb; Ap^R; Km^R	Jongbloed *et al*., 2004
pCCy	pGDL48-derivative containing the *tatCy* gene; 7.5 kb; Ap^R; Km^R	Jongbloed *et al*., 2004
pCACy	pGDL48-derivative containing the *tatAy-tatCy* operon; 7.7 kb; Ap^R; Km^R	Jongbloed *et al*., 2004
B. subtilis		
168	*trpC2*	Kunst *et al*., 1997
tatAy	*trpC2*; *tatAy*::Em; Em^R	Jongbloed *et al*., 2004
tatCy	*trpC2*; *tatCy*::Sp; Sp^R	Jongbloed *et al*., 2000
tatAyCy	*trpC2*; *tatAy*-tatCy::Sp; Sp^R	Jongbloed *et al*., 2002
tatCd	*trpC2*; *tatCd*::Km; Km^R	Jongbloed *et al*., 2000
tatAdCd	*trpC2*; *tatAd-tatCd*::Km; Km^R;	Jongbloed *et al*., 2004
tatAy-tatAd	*trpC2*; *tatAy*::Em; Em^R ; *tatAd*::Km; Km^R	Jongbloed *et al*., 2004
tatCy-tatCd	*trpC2*; *tatCy*::Sp; Sp^R; *tatCd*::Km; Km^R	
total-tat	*trpC2*, *tatAc*::Em; Em^R; *tatAy-tatCy*::Sp; Sp^R; *tatAd-tatCd*::Cm; Cm^R	Jongbloed *et al*., 2002
total-tat_2	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; *tatAc*::Em; Em^R	Jongbloed *et al*., 2004
168 XywbN	*trpC2*; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al*., 2002
tatAyCy XywbN	*trpC2*; *tatAy-tatCy*::Sp; Sp^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al*., 2002
tatAdCd XywbN	*trpC2*; *tatAd-tatCd*::Km; Km^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al*., 2002
total-tat_2 XywbN	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; *tatAc*::Em; Em^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al*., 2002
lytC	*trpC2*; *pheA3*; *purAJ6*; *hisA35*; *metB5*; *lytC*::Cm; Cm^R	Foster, 1992
lytD	*trpC2*; *pheA3*; *purAJ6*; *hisA35*; *metB5*; *lytD*::Cm; Cm^R	Foster, 1992
ywbL	*trpC2*, *ywbL*::pMutin2::Em; Em^R (BFA3211)	Kobayashi *et al*., 2003
ywbM	*trpC2*, *ywbM*::pMutin2::Em; Em^R (BFA3212)	Kobayashi *et al*., 2003
ywbN	*trpC2*, *ywbN*::pMutin2::Em; Em^R (BFA3213)	Kobayashi *et al*., 2003
ywbN XywbN	*trpC2*, *ywbN*::pMutin2::Em; Em^R *amyE*::*xylA-ywbN-myc*: Cm^R	This study

Tris buffer containing 1% Triton X-100. The Micrococcal cell wall in gels was stained with 1% methylene blue. Images were recorded using a Genius:BOX Gel Documentation System and analysed with GeneSnap software (Syngene). Proteomics analyses were performed as previously described [59].

Northern blotting analyses.

For Northern blotting, cells from overnight cultures on LB medium with 1% NaCl were used to inoculate LB medium containing 6% NaCl. Growth was continued till an OD_{600} of ~2 and then the cells were harvested for RNA extraction and blotting

Preparation of total RNA and Northern blot analysis using a specific RNA probe were performed as described previously [30]. The RNA preparations (5 µg per lane) were electrophoretically separated in a 1.2% agarose gel, equal were loaded (not shown). The corresponding chemiluminograph after hybridization of the blotted RNA with a *wprA* probe is depicted in Figure 3. Chemiluminescence was detected using a Lumi-Imager (Roche Diagnostics). Transcript sizes were determined by comparison with an RNA size marker (Fermentas RiboRuler High Range RNA Ladder). The digoxygenin-labeled specific RNA probe was synthesized by *in vitro* transcription using T7 RNA polymerase and a specific PCR product as template. Synthesis of the DNA template was performed by PCR using the following pair of oligonucleotides: wprA-for (5'-ATGAAACGCAGAAAATTCAG-3') and wprA-rev (5'-CTAATACGACTCACTATAGGGAGAAATCAGGCTGTTGACTTGGC-3'). The underlined sequence indicates the T7 promoter region.

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Chapter 5

The Twin-Arginine Translocation (Tat) systems from *Bacillus subtilis* display a conserved mode of complex organisation and similar substrate recognition requirements

James P. Barnett, René van der Ploeg, Robyn T. Eijlander, Anja Nenninger, Sharon Mendel, Rense Rozeboom, Oscar P. Kuipers, Jan Maarten van Dijl, and Colin Robinson

ABSTRACT

The Tat system transports folded proteins across the bacterial plasma membrane. In Gram-negative bacteria, membrane-bound TatABC subunits are all essential for activity, whereas Gram-positive bacteria usually contain only TatAC subunits. In *Bacillus subtilis,* two TatAC-type systems, TatAdCd and TatAyCy, operate in parallel with different substrate specificities. Here, we show that they recognize similar signal peptide determinants. Both systems translocate GFP fused to three distinct *E. coli* Tat signal peptides, namely DmsA, AmiA and MdoD, and mutagenesis of the DmsA signal peptide confirmed that both Tat pathways recognise similar targeting determinants within Tat signals. Although another *E. coli* Tat substrate, TMAO reductase, was translocated by TatAdCd but not by TatAyCy, we conclude that these systems are not predisposed to recognize only specific Tat signal peptides, as suggested by their narrow substrate specificities in *B. subtilis*. We also analyzed complexes involved in the second Tat pathway in *B. subtilis,* TatAyCy. This revealed a discrete TatAyCy complex together with a separate, homogeneous, ~200 kDa TatAy complex. The latter complex differs significantly from the corresponding *E. coli* TatA complexes, pointing to major structural differences between Tat complexes from Gram-negative and Gram-positive organisms. Like TatAd, TatAy is also detectable in the form of massive cytosolic complexes.

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INTRODUCTION

The Twin-arginine translocation (Tat) pathway operates in the bacterial plasma membrane where it serves to transport fully folded proteins into or across the membrane. This process is energised primarily, if not solely, by the proton motive force [1-4], and the Tat pathway functions alongside the well characterised Sec pathway that translocates proteins in an unfolded conformation by an ATP-dependent mechanism. It appears that the Tat pathway exists to facilitate the transport of proteins that fold too tightly or rapidly in the cytosol to be compatible with the Sec pathway. It is also used to translocate proteins that require a cofactor to be inserted in the cytosol prior to transport, such as complex redox enzymes involved in the respiratory chain. Proteins are targeted to the Tat pathway by means of cleavable N-terminal signal sequences that contain a highly conserved twin-arginine motif within the consensus sequence (S/T)-R-R-x-F-L-K) [5-7]. At least three distinct targeting determinants within this motif have been shown to be important for Tat translocation in bacteria [8].

Gram-negative bacteria contain three essential Tat components, namely the integral membrane proteins TatA, TatB and TatC. These have molecular masses of 10 kDa, 18 kDa and 30 kDa, respectively, in *E. coli* (which is by far the best studied bacterial Tat system). The genes encoding these three proteins are coexpressed in an operon with a fourth *tat* gene, *tatD,* which is not involved in the Tat pathway [9]. A fifth *tat* gene, *tatE,* is also present in *E. coli* and expressed elsewhere in the genome. This gene is thought to be a cryptic gene duplication of *tatA* as it can functionally complement a *tatA* null mutant. The *tatE* gene is expressed at a very low level relative to the *tatA* gene and is not thought to play any significant role in the Tat pathway [10,11].

The three essential Tat components form two types of complexes within the plasma membrane: a substrate-binding TatABC complex of around 370 kDa, in which TatB and TatC are the critical components, and a series of separate TatA complexes that vary in size from less than 100 kDa to well over 500 kDa [12,13]. It has been suggested that these TatA complexes are involved in the formation of pores through which Tat substrates are translocated [14], with the size variation perhaps linked to the need to transport substrates of differing size. Recently, some doubt has been cast on the functional significance of the size variation of the TatA oligomers, since mutant TatA proteins form such oligomers even in the absence of Tat-specific protein translocation [15].

The Tat systems of Gram-positive bacteria exhibit interesting differences to those of Gram-negative bacteria, the most striking of which is the absence of a TatB component in virtually all species. Some Gram-positive bacteria, such as *Bacillus subtilis,* also contain multiple Tat pathways that operate in parallel with differing substrate specificities [16]. *B. subtilis* is a harmless soil dwelling bacterium that contains three *tatA* genes, denoted *tatAd*, *tatAy* and *tatAc*, and two *tatC* genes denoted *tatCd* and *tatCy*. The *tatAd* gene is expressed in an operon with *tatCd* and these two components form a minimal Tat translocase responsible for the translocation of the substrate PhoD. The *phoD* gene is expressed upstream of the *tatAd/Cd* genes and this operon is expressed under phosphate-limited conditions. PhoD is the only known substrate of the TatAdCd system [17-19]. The protein has phosphodiesterase and alkaline phosphatase activity, and PhoD is targeted to the cell wall, where it is involved in the release of inorganic phosphate [20].

The absence of a TatB component led to the idea that the TatAd protein may be bifunctional, fulfilling the roles of both TatA and TatB of *E. coli* [16]. We confirmed this point in a recent study by showing that TatAd could indeed complement both the *E. coli tatA/E* and *tatB* null mutant strains [21]. The TatAd and TatCd proteins were also shown to form two types of complexes within the membrane: a TatAdCd complex that is significantly smaller than its *E. coli* counterpart (~230 kDa as judged by Blue-native PAGE) and a homogeneous TatAd complex (~160 kDa as judged by gel filtration) that does not exhibit the same size variation as *E. coli* TatA complexes [21].

The *tatAy* and *tatCy* genes are coexpressed in an operon to form a second minimal Tat translocation pathway in *B. subtilis* [17]. This operon is constitutively expressed and only a single substrate has been identified for this pathway: YwbN, a heme-containing DyP-type peroxidase. The third *tatA* gene of *B. subtilis, tatAc,* is not expressed with any other Tat components and its contribution to the Tat pathway is not known [17,18,22]. Recently, two other *B. subtilis* Tat substrates (QcrA and YkuE) have been identified using a facile reporter system, although their preferred Tat pathway for secretion in *B. subtilis* is not yet known [23].

In this study we have investigated the substrate specificities of the two Tat pathways of *B. subtilis* in order to determine whether they are predisposed to recognize specific Tat signals. We show that both the TatAdCd and TatAyCy systems recognise surprisingly similar

targeting determinants despite their distinct substrate specificities within *B. subtilis*. In addition, we show that, like TatAdCd, the TatAyCy system consists of two types of complexes within the membrane, a TatAyCy complex and a separate TatAy complex that resemble more closely the TatAdCd and TatAd complexes than the known *E. coli* Tat complexes. This observed homogeneity of TatA complexes in *B. subtilis* suggests that this may be a general feature of TatA complexes in Gram-positive bacteria, and a major difference compared to Gram-negative species. A somewhat controversial aspect of *B. subtilis* Tat studies has been the identification of a cytosolic species of TatAd that has been shown to bind the substrate PhoD [24]. This led to the suggestion that TatAd binds its substrate in the cytosol and acts as a guidance factor, targeting substrate molecules to membrane-localised TatCd by a mechanism that would be completely different to the current *E. coli* model [25]. We therefore considered it important to test for the presence of cytosolic TatAy. We show that TatAy does indeed have a cytosolic as well as a membrane-associated localisation, and the possible significance of this cytosolic TatA is discussed.

RESULTS

TatAyCy is active in *E. coli* and able to recognize three different *E. coli* Tat signal peptides.

TatAdCd has previously been shown to be active in *E. coli* and able to export fusion proteins comprising the signal peptides of TorA and DmsA linked to GFP [8]. Separate TatAdCd and TatAd complexes were characterized and shown to be very different from their *E.* coli counterparts. However, TatAdCd is an exceptional Tat system. In order to understand Gram-positive Tat systems in a more general sense, and simultaneously probe the basis for the observed strict substrate specificities of TatAdCd and TatAyCy in *B. subtilis*, we analyzed the TatAyCy system in terms of substrate specificity and complex organisation. A key aim was to probe the mechanism of the TatAyCy system in the light of suggestions that Gram-positive Tat systems may operate in a fundamentally different manner to those of Gram-negative organisms.

In order to directly compare the substrate specificities of the TatAdCd and TatAyCy systems, we first tested whether overexpressed TatAyCy is likewise able to form an active translocation system in *E. coli*, with the aim of analyzing the abilities of the two systems to transport a range of

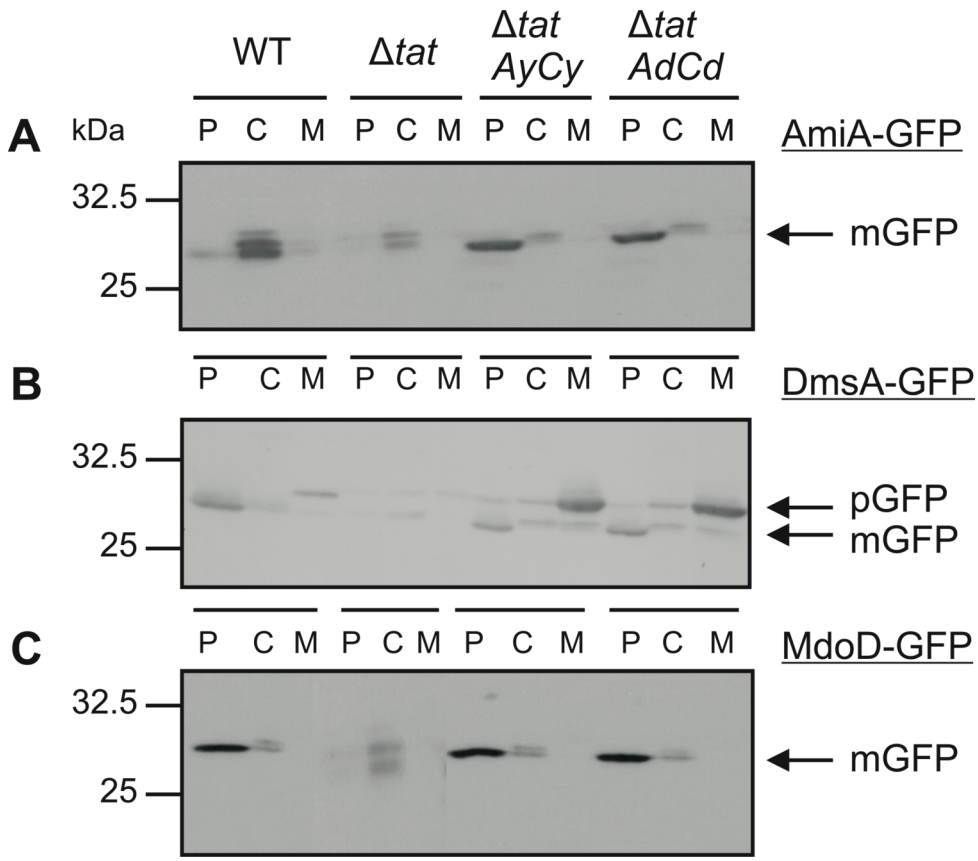

Figure 1. Expression of *B. subtilis* TatAyCy and TatAdCd leads to export of AmiA-GFP, DmsA-GFP and MdoD-GFP in *E. coli*. Constructs comprising the **(A)** AmiA, **(B)** DmsA or **(C)** MdoD signal peptide (SP) linked to GFP (TatSP-GFP) were expressed from the pBAD24 plasmid in WT MC4100 cells, Δ*tatABCDE* cells (Δtat), and Δ*tatABCDE* cells expressing *B. subtilis* TatAyCy or TatAdCd (using the compatible pEXT22 plasmid). Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP antibodies. Mobilities of the precursor forms and mature size GFP are indicated (pGFP, mGFP). Mobilities of molecular weight markers are given on the left (in kDa).

substrates. The *tatAyCy* genes were overexpressed in an *E. coli tat* null *(ΔtatABCDE)* mutant on the pEXT22 plasmid alongside one of three heterologous Tat substrates expressed on the compatible pBAD24 plasmid. The substrates comprised green fluorescent protein (GFP) fused to the Tat signal peptides of *E. coli* AmiA, MdoD, or DmsA. In addition, wild-type *E. coli* (MC4100) cells expressing the substrate were used as a positive control for export and *ΔtatABCDE* cells were used as a negative control. Following expression from both plasmids, cells were fractionated into periplasm (P), cytoplasm (C), and membrane (M) fractions and analyzed by immunoblotting with anti-GFP antibodies. Figure 1A shows that AmiA-GFP is exported by wild-type (WT) cells, with mature-size GFP detected in the periplasmic fraction (P). No periplasmic band was observed in the *tat* null mutant

strain as expected, but most of the AmiA-GFP is exported when either TatAyCy or TatAdCd is expressed in the *tat* null background, with strong mature-size GFP (mGFP) signals in the periplasmic samples. Indeed, export is more efficient than with wild type cells (where the periplasmic mature band is rather weak) but this may reflect the fact that the *tatAdCd* genes, or the *tatAyCy* genes, are overexpressed compared to wild type cells. This demonstrates for the first time that TatAyCy is active in *E. coli*. The cytosolic fractions contain bands caused by proteolytic cleavage of the precursor protein as observed previously (it should also be noted that this assay is not quantitative and the amount of protein detected is variable, again as described previously [8]). Essentially the same results were obtained with the two other substrates tested; DmsA-GFP export assays are shown in Figure 1B and MdoD-GFP assays are shown in Figure 1C. Both substrates are exported by WT *E. coli* cells, and by cells overexpressing TatAyCy and TatAdCd. MdoD-GFP, in particular, is an excellent substrate for these studies with the vast majority exported by both *B. subtilis* systems as well as by the TatABC system in wild-type *E. coli* cells. DmsA-GFP is exported more efficiently in wild-type cells than in TatAdCd- or TatAyCy-expressing cells, with a greater accumulation of precursor protein (pGFP) evident in the latter cases. The precursor protein is mostly found in the membrane fraction, in agreement with earlier studies [26] in which a Tat signal peptide-GFP fusion was found to accumulate strongly with the membrane if not exported (much of the membrane-bound GFP was incorrectly folded, suggesting a non-specific interaction rather than a specific interaction with the translocon). Nevertheless, these cells clearly export DmsA-GFP with a clear periplasmic mature-size band apparent in both cases. No export of any substrate was observed in the *tat* null mutant strain, and most of the cytoplasmic protein is degraded as observed in a previous study involving the use of DmsA-GFP [27]. This shows that the two *B. subtilis* Tat systems recognise all three Tat signal peptides, despite exhibiting markedly different substrate specificities in *B. subtilis*.

TatAyCy is unable to transport some Tat substrates.

We also tested whether TatAyCy can transport a natural *E. coli* Tat substrate, TMAO reductase (TorA). TorA is one of the largest known Tat substrates (90 kDa), and is required for anaerobic growth on minimal TMAO and glycerol media. Well-established export assays have been described for TorA transport and we have shown previously that the TatAdCd system can efficiently export this substrate when expressed in *E. coli tat* mutant cells [21].

Figure 2A shows a TorA export assay in which TorA activity is detected using a native polyacrylamide gel involving a methyl-viologen based reduction that results in the clearing of gel turbidity in the presence of TMAO reductase and substrate. The left hand panel shows WT *E. coli* as a positive control. A white band is clearly present in the periplasmic (P) lane, indicating export as expected. Some TorA activity is also apparent in the cytosolic (C) fraction as has been observed previously. No activity was detected in the membrane (M) fraction. As a negative control we also ran samples from the *E. coli tat* mutant strain *(ΔtatABCDE),* and TorA activity is exclusively cytosolic in these cells. As a second positive control we analyzed cell fractions from *E. coli ΔtatABCDE* cells expressing TatAdCd from the pBAD24 plasmid. As described previously, TorA is exported to the periplasm with high efficiency. In contrast, the right hand panel shows samples from *ΔtatABCDE* cells expressing TatAyCy, and the data show that TorA activity is localised exclusively in the cytoplasmic fraction, with no export apparent. Thus, although both TatAdCd and TatAyCy were able to translocate the three substrates tested above, not all substrates are compatible with the TatAyCy system and a degree of substrate specificity is observed between the two pathways.

Given that TatAyCy cannot export TorA, this could be due to an inability to (i) recognize the TorA signal peptide or (ii) handle the mature TorA protein. We addressed the first possibility by expressing the *tatAyCy* genes on the pEXT22 plasmid as described above, together with a construct comprising the TorA signal peptide fused to GFP on the pBAD24 plasmid; the data are shown in Figure 2B. In control tests, TorA-GFP is exported to the periplasm (P) in WT *E. coli* cells as shown before [21]. In the *E. coli tat* mutant strain *(Δtat)* no band is apparent in the periplasmic fraction, again as expected. We have shown previously [21] that TatAdCd is able to efficiently translocate TorA-GFP, and as an additional positive control we expressed TatAdCd from the pEXT22 plasmid together with the pBAD-TorA-GFP construct in the *E. coli ΔtatABCDE* strain. Reproducing earlier findings, we observe mature size GFP in the periplasmic fraction, confirming export. Finally, the right hand panel shows that TatAyCy-expressing cells are unable to transport TorA-GFP, as no GFP is detectable in the periplasmic fraction. The TatAyCy system is thus unable to recognise the TorA signal peptide (since TatAyCy can transport GFP when other Tat signals are attached (Fig. 1). This may explain the failure to export the native TorA precursor protein, but we should point out that our data do not exclude the possibility that TatAyCy may also be incapable of handling the TorA mature protein.

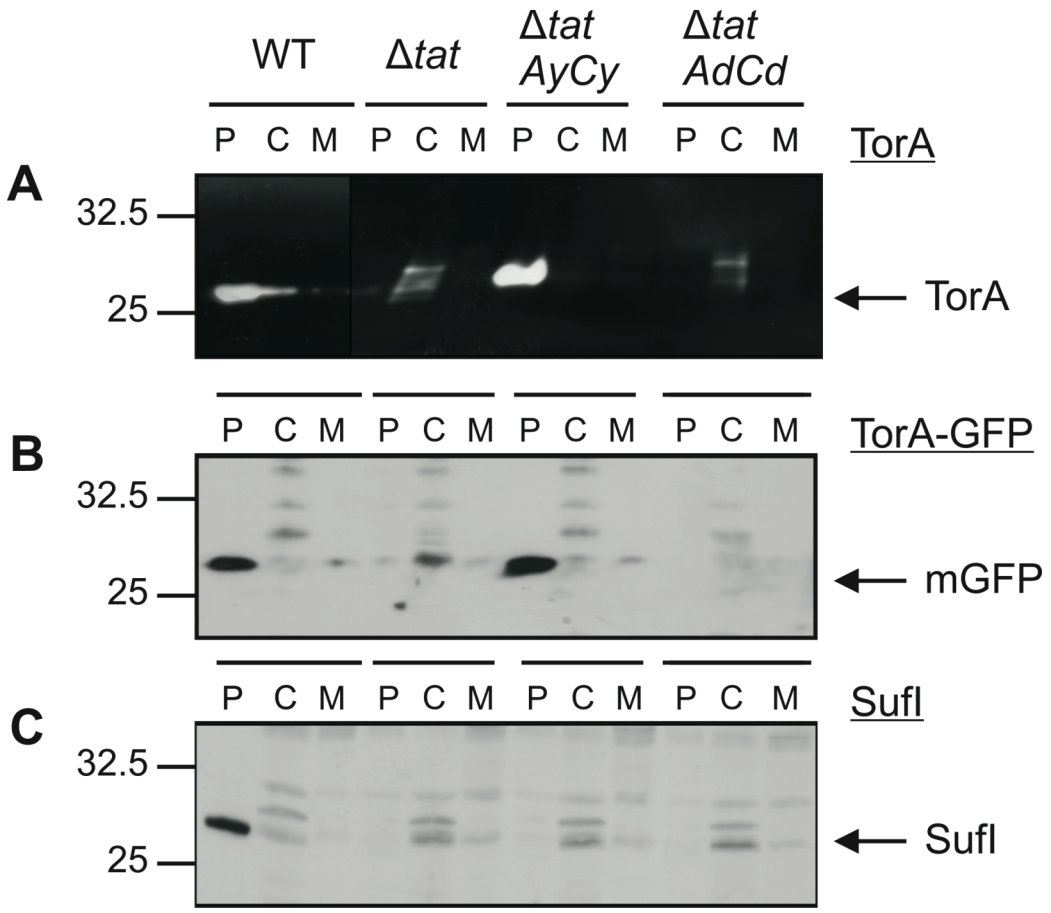

Figure 2. TMAO reductase, TorA-GFP and SufI are not transported by TatAyCy. (A) native polyacrylamide gel stained for TMAO reductase (TorA) activity. Membrane, cytoplasmic and periplasmic fractions (M, C, and P) were prepared and analyzed from WT MC4100 cells, Δ*tatABCDE* cells (Δtat), and Δ*tatABCDE* cells expressing *B. subtilis* TatAyCy or TatAdCd from plasmids pEXT-AyCy and pEXT-AdCd. Mobility of active TorA is indicated. **(B)** a construct comprising the TorA signal peptide fused to GFP (TorA-GFP) was expressed from plasmid pBAD24 in WT MC4100 cells, Δ*tatABCDE* cells (Δtat), and Δ*tatABCDE* cells expressing *B. subtilis* TatAyCy or TatAdCd (using the compatible pEXT22 plasmid). Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP antibodies. Mature size GFP is indicated (mGFP). **(C)** Cell fractions prepared from WT MC4100 cells, Δ*tatABCDE* cells (Δtat), and Δ*tatABCDE* cells expressing *B. subtilis* TatAyCy or TatAdCd from plasmids pBAyCy and pBAdCd were also immunoblotted using specific anti-SufI antibodies. The mobility of mature SufI is indicated and molecular weight markers are indicated on the left (in kDa).

We finally tested one other *E. coli* Tat substrate for export by TatAyCy. SufI is a 50 kDa *E. coli* periplasmic protein thought to play a role in cell division [28]. We have shown previously that SufI cannot be exported by the TatAdCd pathway [21], and Fig. 2C shows tests to determine whether TatAyCy can export this substrate. The left hand panel shows fractions from *E. coli* WT cells, with mature size SufI detected in the periplasmic (P) fraction. No such band was observed in the periplasm of *E. coli ΔtatABCDE* cells as was expected. The remaining panels show that neither

TatAdCd nor TatAyCy (both expressed from the pBAD plasmid) are able to support export, with no SufI detectable in the periplasm. In summary, both the TatAdCd and TatAyCy pathways are active in *E. coli* and able to recognise several different *E. coli* Tat signal peptides, but not all substrates are compatible and a degree of substrate specificity is evident between the two pathways.

The TatAyCy and TatAdCd pathways recognize the same targeting determinants within signal peptides.

We have previously shown, by site directed mutagenesis of Tat signal peptides, that the *E. coli* TatABC and *B. subtilis* TatAdCd systems recognise similar targeting determinants within the signal peptides of their substrates [8]. Within the DmsA signal peptide we found that the twin arginine motif, the -1 serine residue and the +2 leucine residue (with respect to the twin-arginine motif) are all important for efficient translocation by TatAdCd and *E. coli* TatABC. In order to determine if this was also true for TatAyCy we tested the ability of TatAyCy to export the DmsA-GFP fusion protein containing specific mutations within the signal peptide. We initially focused on the most conserved residues within the consensus motif, the twin arginines. Figure 3 shows that the non-mutated DmsA-GFP construct (WT) is exported in these cells and processed to the mature size in the periplasm (P). A considerable amount of mature-size protein is also found in the cytoplasm (C), due to non-specific proteolysis of the signal peptide [8]. The data also show that substitution of both arginines to lysines (KK) results in a complete block in export, as no mature sized GFP band is evident in the periplasmic sample (P). Substitution of single arginine residues to lysine (RK, and KR) results in a level of export that is so low it is barely detectable using our assay system. Only a very weak mature size GFP band is observed in the periplasmic fraction, confirming the importance of these two residues for export. We also tested the importance of the +2 leucine residue (Leu19) within the consensus motif by testing for export of the DmsA-GFP fusion carrying the L19A, L19D, and L19F mutations (Fig. 3B). In results similar to those obtained with TatAdCd, we find that the L19A and L19D mutations result in a complete block in export while the L19F mutant allows a low but detectable level of translocation to occur (indicated by the presence of mature size GFP in the periplasmic sample lane). Finally, we tested one other residue, the highly conserved -1 serine (Ser15) by replacing it with alanine. We find that this substitution allows for only a very low level of translocation activity as indicated by a weak mature size GFP band in the periplasmic sample. Again, this result is similar to that obtained

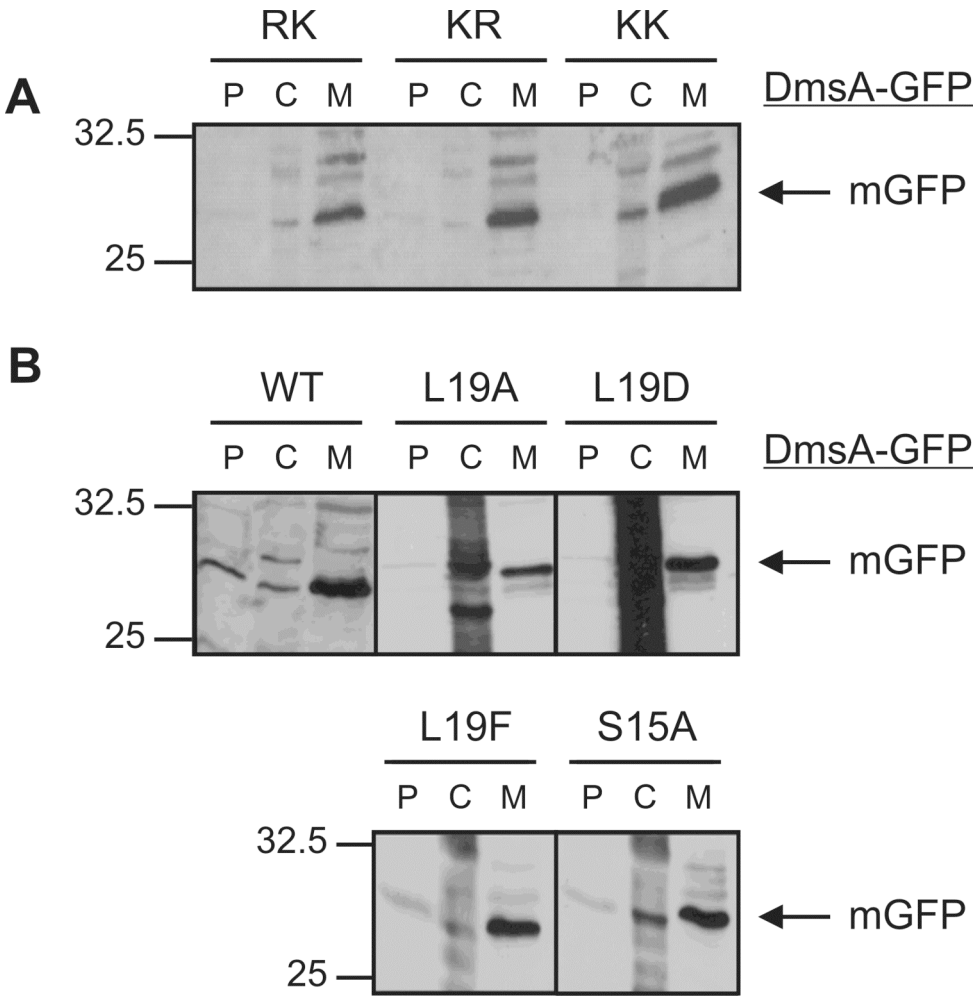

Figure 3. Mutagenesis of the DmsA signal peptide. Constructs comprising the DmsA-signal peptide fused to GFP carrying different mutations (as indicated) were expressed from the pBAD24 plasmid along with TatAyCy expressed from the compatible pEXT22 plasmid in *E. coli* Δ*tatABCDE* cells. Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP antibodies. Mature size GFP is indicated (mGFP) and the mobilties of molecular weight markers (in kDa) are indicated on the left.

with the TatAdCd system using the same mutated DmsA-GFP. We conclude that the TatAyCy and TatAdCd systems are not only capable of recognising a very similar set of Tat signal peptides, but they also recognise the same conserved targeting determinants within the Tat consensus motif that are indispensable for productive protein translocation.

Several of the mutated precursor proteins associate strongly with the membrane in the absence of efficient export. It is possible that the proteins are associating with the translocon but failing to be properly translocated. However, as pointed out above, we have also observed very strong membrane-association of precursor proteins in other studies and we favour the

explanation that the membrane-assocation is non-specific [26].

Characterization of separate TatAyCy and TatAy complexes formed during overexpression of the *tatAyCy* genes.

In order to study the TatAyCy translocase complexes, *E. coli ΔtatABCDE* cells expressing TatAyCy-strep (with a Strep-II tag fused to the C-terminus of TatCy) from the plasmid pBAyCys were fractionated and membranes were isolated. Total membranes were solubilised in 2% digitonin and subjected to streptactin affinity chromatography as described in Materials and Methods. All column fractions were immunoblotted using antibodies to the Strep-II tag on TatCy, and to TatAy (Fig. 4, top panel). Using the anti-Strep antibody, a proportion of TatCy-strep was detectable in the column wash fractions, but most of the protein bound to the column. The TatCy-strep was then specifically eluted from the column across elution fractions 2-5 with a clear peak in fraction 3 (arrowed). A corresponding band was present in the same peak elution fraction in the TatAy immunoblot, indicating the presence of a TatAyCy complex. The vast majority of the TatAy protein did not bind the column and was detected in the first few column wash fractions, indicating the presence of a separate TatAy complex. To confirm the association of TatAy and TatCy in a complex, the peak fractions from the first column were pooled and run on a second streptactin column (Fig. 4, lower panel). The column was washed and eluted in the same manner and the data show that the majority of both subunits co-elute in the elution fractions. This confirms the presence of a TatAyCy complex. In summary, the combined data clearly point to the presence of separate TatAyCy and TatAy complexes, and the key point is that this two-complex organisation is a common feature of all Tat systems analysed in this way to date [12,21].

Gel filtration chromatography reveals a TatAyCy complex of ~200 kDa.

Apart from the absence of a TatB component, the earlier study on the TatAdCd system revealed a major difference from the *E. coli* TatABC system in that TatAd is present as a small, highly homogeneous complex [21]. The corresponding *E. coli* TatA complex is remarkably heterogeneous, with an average size far greater than the TatAd complex, and this raises the possibility that the TatAd complex is atypical, with its restricted size distribution perhaps related to the narrow substrate specificity. To characterise a second Gram-positive Tat system in this respect, we examined the size and characteristics of both the TatAyCy and TatAy complexes.

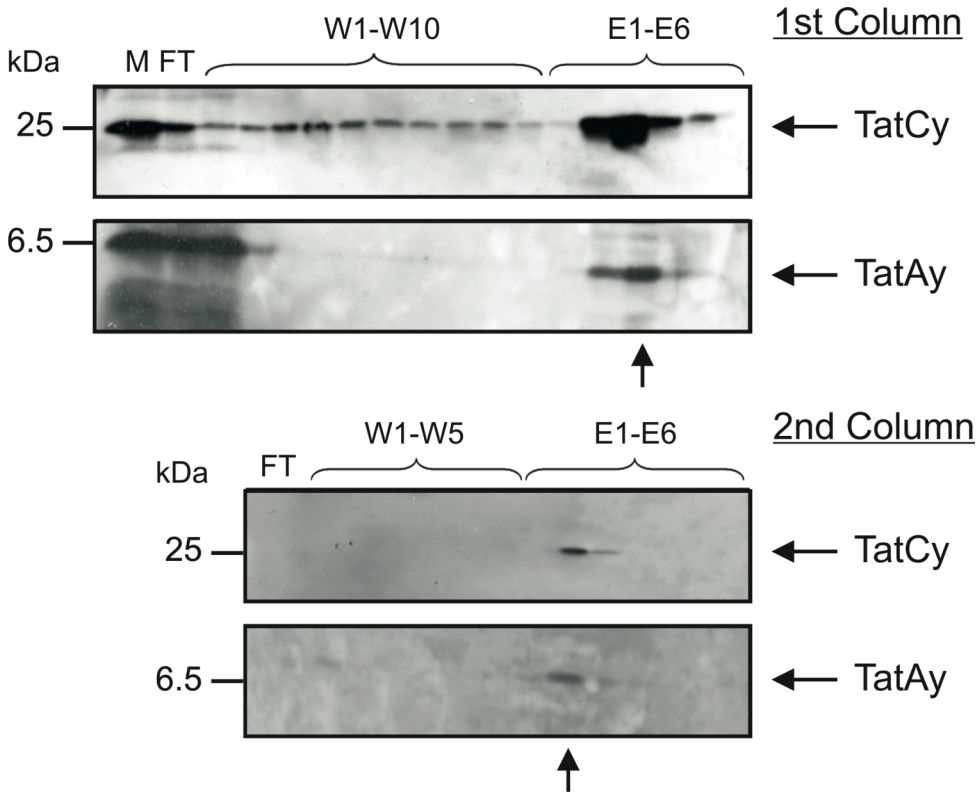

Figure 4. Separation of distinct TatAyCy membrane-bound complexes. Membranes were prepared from Δ*tatABCDE* cells expressing *B. subtilis* TatAyCy (from plasmid pBAyCys), solubilised in digitonin and applied directly to a Streptactin affinity column as described in Materials and Methods. All column fractions were immunoblotted using antibodies against the strep-II tag on TatCy and to TatAy. Peak TatCy- and TatAy-containing fractions in the elution series (E2-4) were pooled and re-run on a second column, which was processed in an identical manner. Whole membranes (M), column flow through (FT), wash fractions (W1-10), and elution fractions (E1-6) are all indicated. Mobility of TatCy-strep and TatAy are indicated on the right. Molecular weight markers are indicated on the left.

For analysis of the isolated TatAyCy complex, the peak elution fractions from the streptactin column (see above) were pooled, concentrated and applied to a calibrated Superose-6 gel filtration column. All column elution fractions were immunoblotted using antibodies to the Strep-II tag on TatCy or to TatAy (Fig. 5A). The immunoblots show TatCy to elute in fractions 20-28 with a peak in fraction 25. A small amount of TatAy co-elutes with TatCy, confirming that these two components are present as a stable complex. Only a very small proportion of the TatAy protein present in the plasma membrane is found in this complex as shown above using affinity chromatography. This is reflected by the weakness of the band that is detectable in the TatAy immunoblot. The peak elution fractions were analysed by densitometry and band intensity was plotted against fraction number (Fig. 5B). The data show that the complex is eluting as a relatively

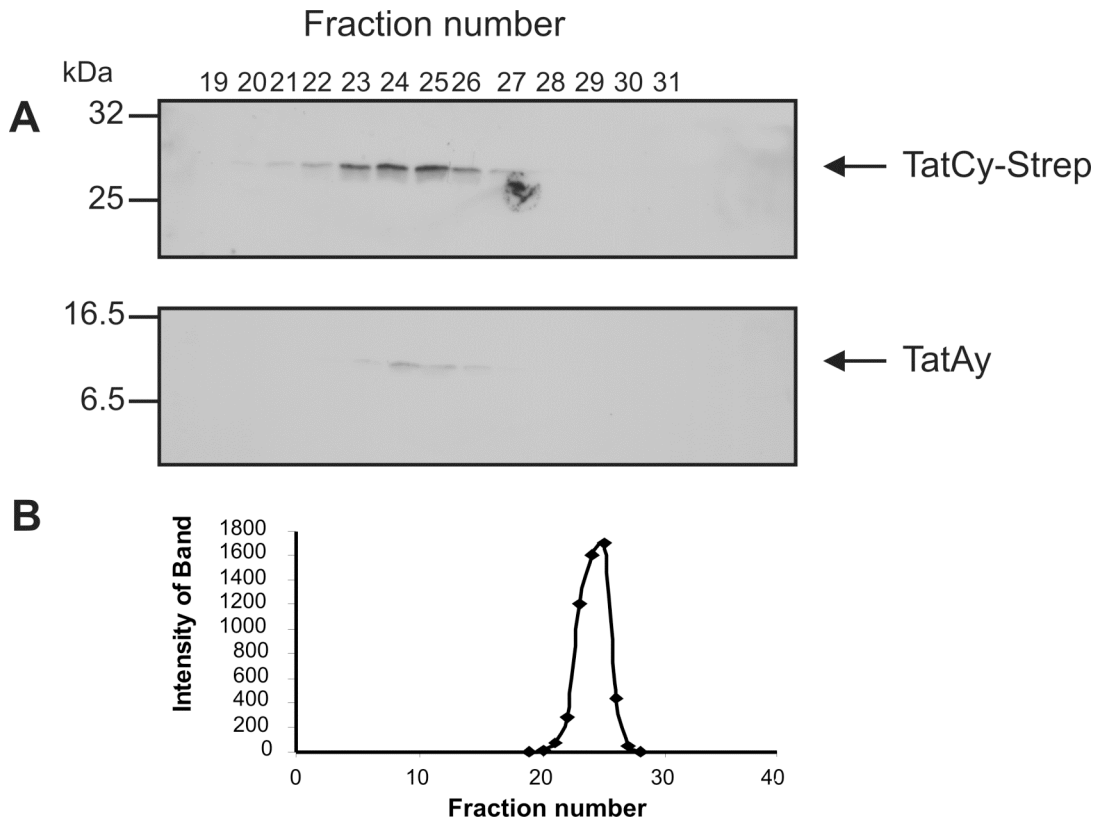

Figure 5. Purified TatAyCy is a discrete 200kDa complex. (A) Affinity purified TatAyCys was applied to a Superose-6 gel filtration column as described in materials and methods. Peak elution fractions (19-31) were immunoblotted using antibodies against the strep-II tag on TatCy and to TatAy. Mobility of TatCy-strep and TatAy are shown on the right. Molecular weight markers (in kDa) are indicated on the left. **(B)** The TatCy immunoblot was analysed by densitometry and intensities of bands plotted against fraction number. The column was calibrated using a set of protein standards of known molecular weight, namely thyroglobin (669 kDa), ferritin (440 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7kDa).

tight peak (suggesting that TatAyCy is rather homogeneous) and calibration of the column shows the complex to be ~200 kDa in size, significantly smaller than the *E. coli* TatABC complex (600 kDa by gel filtration chromatography [13]) and even smaller than the TatAdCd complex (350 kDa by gel filtration chromatography [21]). These size estimates are influenced by the size of the detergent micelle and the true sizes of the protein complexes are likely to be smaller.

Membrane-bound TatAy complex is small and homogeneous (~200 kDa) whereas cytosolic TatAy forms large complexes or aggregates (~5 MDa).

The TatAy complex was analyzed in a similar manner, but in this case we studied the complex after isolation from both the membrane and cytosol fractions. Recent work on the TatAdCd pathway of *B. subtilis* has shown that TatAd is in the cytosol as well as the plasma membrane, and the cytosolic form has been proposed to

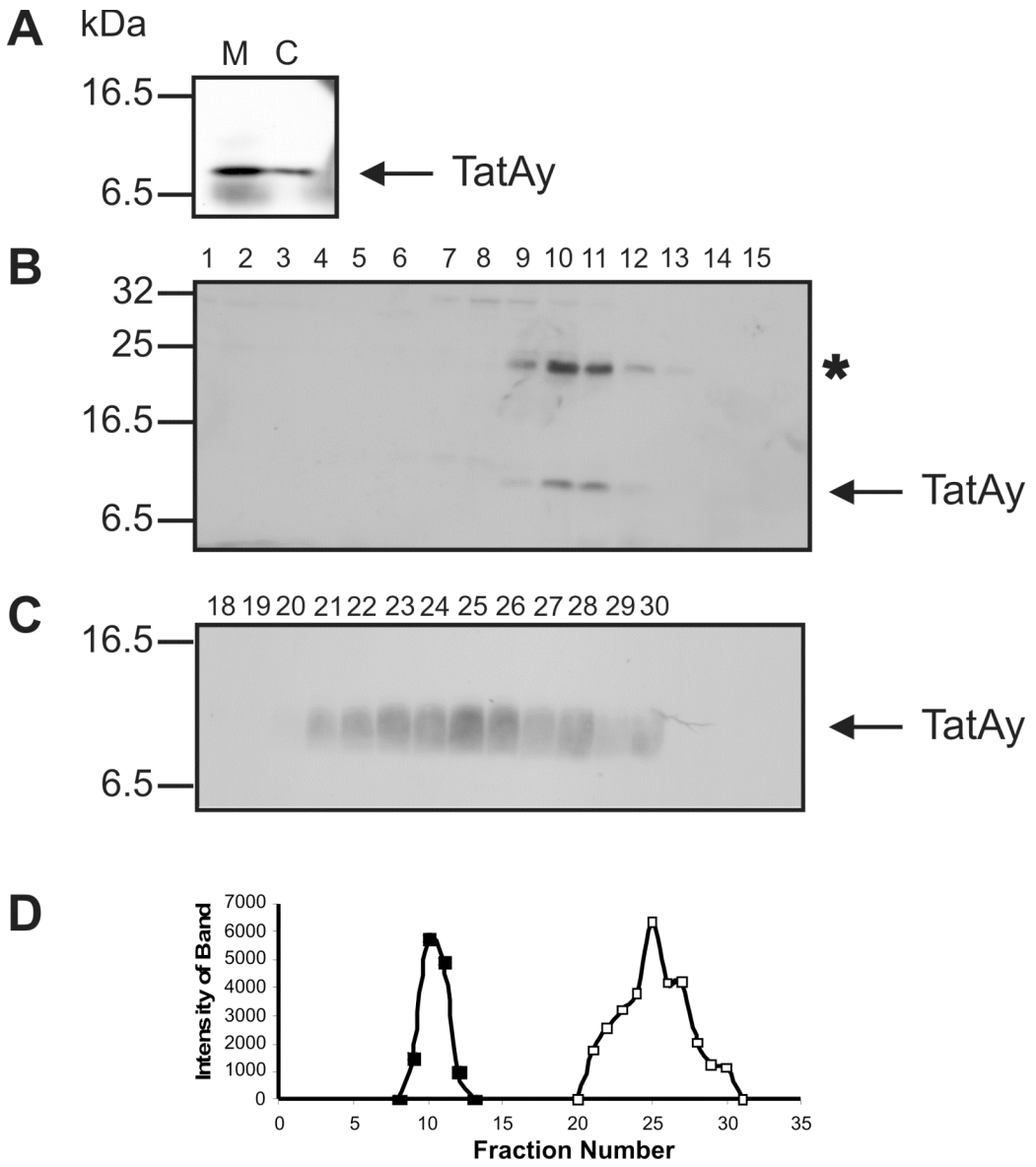

Figure 6. TatAy forms a ~200 kDa complex within the plasma membrane and large aggregates within the cytosol. **(A)** membrane (M) and cytosolic (C) fractions of *E. coli* Δ*tatABCDE* cells overexpressing TatAyCys were immunoblotted with anti TatAy antibodies. Molecular weight markers (in kDa) are shown on the left and mobility of TatAy on the right. **(B)** the cytosolic fraction of *E. coli* Δ*tatABCDE* cells overexpressing TatAyCy was applied directly to a superose-6 HR gel filtration column in the absence of detergent and peak elution fractions (1-15) were immunoblotted with anti-TatAy antibodies. Molecular weight markers are indicated on the left. Mobility of TatAy is shown. A slower running band in lanes corresponding to the TatAy elution is indicated with an asterisk. **(C)** Streptactin column flow-through and wash fractions containing free TatAy complexes isolated from membranes from TatAyCy overexpressing cells were also subjected to gel filtration chromatography and peak elution fractions (18-33) were immunoblotted with anti-TatAy antibodies. Mobilities of molecular weight markers (in kDa) are indicated on the left and mobility of TatAy shown on the right. **(D)** immunoblots of all elution fractions of membrane-localised and cytosolic TatAy were analysed by densitometry. Intensities of the bands were plotted against fraction number. The column was calibrated using standards of known molecular weight as detailed in Figure 5. Membrane localised TatAy is indicated with open squares and cytosolic TatAy is indicated with filled squares.

act as the initial receptor for substrate [24]. We first sought to determine whether TatAy also displays this dual localisation when expressed in *E. coli.* For this purpose, we introduced plasmid pBAyCys in *E. coli ΔtatABCDE* cells and then cytosolic (C) and membrane (M) samples were analysed using specific TatAy antibodies (Fig. 6A). The data show that TatAy is indeed present in both membrane and cytosolic fractions. We also analysed the size and homogeneity of the cytosolic and membrane-associated TatAy complexes, using the Superose-6 column as above. This column has a separation range of 5 kDa to 5 MDa. We found that cytosolic TatAy eluted over fractions 8-12 with a peak in fraction 10 (Fig. 6B), which equates to a size of ~10 MDa as determined from the calibration curve prepared using the markers detailed in Figure 6. This value is above the theoretical maximum size range given for this column, which prevents us from making an accurate determination of the complex size. Nevertheless, the data demonstrate that the cytosolic TatAy complex is ca. 5 MDa in size (or larger). It is therefore likely that the cytosolic TatAy is forming large complexes or aggregates in the same way as cytosolic TatAd has been found to do previously [21]. A slower migrating band is also present in the immunoblot that follows the elution pattern of monomeric TatAy (indicated with *). This band may represent a dimer or trimer of cytosolic TatAy.

In contrast, membrane-localised TatAy eluted across fractions 20-30 with a peak in fraction 25 (Fig. 6C), corresponding to a size of ca. 200 kDa. Finally, immunoblots were analysed by densitometry and the intensity of the bands was plotted against the fraction number, with cytosolic TatAy indicated by filled squares and membrane-bound TatAy by open squares. (Fig. 6D). The data confirm that the membrane-bound TatAy complex is much smaller than the cytosolic TatAy complex; it is also far more homogeneous when compared with *E. coli* TatA complexes that were isolated and run under exactly the same conditions [12,14].

DISCUSSION

Most studies on bacterial Tat pathways have been carried out on *E. coli*, with broadly similar results obtained in studies carried out using other Gram-negative bacteria [13]. These studies have identified separate TatABC and TatA complexes in the plasma membrane, with the latter varying in size from less than 100 kDa to over 500 kDa [12,14]. Current data point to a model where, following substrate binding to the TatABC complex [29], the TatA complex is recruited to form the full

translocation system. Gram-positive organisms usually lack a TatB component, and this suggests that the TatA component is bifunctional, fulfilling the roles of both *E. coli* TatA and TatB. This has been experimentally confirmed for TatAd from *B. subtilis* [16]. However, studies on the TatAdCd system also revealed other differences, especially concerning the nature of the Tat complexes. In this study, we have sought to study the second Tat complex of *B. subtilis*, TatAyCy, to determine similarities and differences that may shed new light on the substrate specificity displayed by both complexes in their host organism

In our previous work, gel filtration chromatography using the detergent digitonin gave a size estimate of ~600 kDa for *E. coli* TatABC [22] but just 350 kDa for TatAdCd [21]. In this study we show the TatAyCy complex to be even smaller with a size estimate of just 200 kDa. It thus appears that a clear difference may exist between the Tat complexes of Gram-negative and Gram-positive bacteria in terms of the size of the TatC-containing substrate-binding complex. Some of the difference may stem from the absence of a TatB component, but the complexes may well contain differing numbers of TatC containing domains and this important question merits further attention. The most notable characteristic of the TatAd complex is that it displays none of the heterogeneity found among *E. coli* TatA complexes [12,14]. Here, we show that the TatAy complex is both small and homogeneous with an estimated size (using gel filtration chromatography) of ~200 kDa, which is again even smaller than the TatAd complex (estimated to be ~270 kDa under the same conditions; ref [21]). The key point is that the TatAy complex, like the TatAd complex, is relatively homogeneous. This provides the first indication of a major general difference between TatA complexes of Gram-positive and Gram-negative bacteria. In this context, it is interesting that the *B. subtilis* Tat systems are capable of transporting a variety of substrates with very different sizes. This finding suggests that there is no strict correlation between TatA heterogeneity and substrate sizes.

Our data also have relevance for the biological roles of the TatAdCd and TatAyCy systems in *B. subtilis*. Here, we have shown that both the TatAdCd and TatAyCy systems are able to recognize and transport a wide variety of heterologous signal peptides, all of which differ widely in primary sequence. Clearly, the TatAdCd system is not predisposed to interact with only the PhoD signal peptide. Moreover, the mutagenesis studies strongly suggest that the signal peptide determinants shown to be recognized by TatAdCd [8] are equally important for productive interaction

with the TatAyCy system. This raises the question of why two distinct Tat systems are present in *B. subtilis*, and this question remains open. One possibility would be that the TatAdCd system provides additional capacity for Tat-dependent protein export under conditions of phosphate starvation that lead to a massive induction of PhoD synthesis.

A controversial aspect of studies into the TatAdCd system of *B. subtilis* was the identification of a soluble substrate-binding species of TatAd in the cytoplasm [24]. This led to the idea that the substrate first interacts with cytosolic TatAd before targeting to the membrane-localised TatCd component [25]. This would imply that completely different mechanisms might be operating in *B. subtilis* and *E. coli*. We recently found that the TatAdCd pathway was active in an *E. coli* background and able to translocate the *E. coli* Tat substrate TMAO reductase (TorA) [21]. In *E. coli,* TorA has its own dedicated cytosolic chaperone TorD, which binds strongly to its signal peptide prior to its recognition by the membrane localised TatABC substrate binding complex [30]. The fact that TatAdCd can translocate TorA in *E. coli* suggested that in fact this Tat system is operating in a manner more closely resembling the *E. coli* model. We further found that cytosolic TatAd, present following overexpression of TatAdCd in *E. coli*, was forming large complexes or aggregates for which a possible role in the translocation process is difficult to assess unambiguously [21]. We therefore considered it important to test for the presence of cytosolic TatAy in the present study. We did indeed find that alongside its membrane localisation, TatAy was present as a soluble species in the cytosol. We also found that, like cytosolic TatAd, TatAy forms very large complexes or aggregates. The functional significance of this pool of cytosolic TatA is not clear. A recent study has found that *E. coli* TatA can also be found in the cytoplasm where it forms large homooligomeric complexes in tube-like structures [31]. The presence of large soluble TatA complexes in *E. coli* suggests that the Tat systems of Gram-positive and Gram-negative bacteria may be more similar than previously thought. This fits well with our observation that the *E. coli* and *B. subtilis* Tat systems are capable of translocating a similar set of substrates, but makes the differences we observe between the membrane localised Tat complexes of *E. coli* and *B. subtilis* all the more intriguing. However, the ability of *E. coli* to export such a range of substrates, when co-expressing either the native Tat system ot either *B. subtilis* Tat system, provides a powerful tool to investigate both the structures and functions of the different Tat complexes.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

All strains and plasmids used are listed in Table I. *E. coli* MC4100 [32] was used as the parental strain. The Δ*tatABCDE* strain [11] has been described previously. Arabinose-resistant derivatives were used as described previously. *E. coli* was grown aerobically in Luria Bertani broth (LB) at 37 °C. *E. coli* was grown anaerobically in LB supplemented with 0.5% glycerol, 0.5% trimethylamine *N*-oxide (TMAO), and 1

Table I. Plasmids and Strains

Plasmids	Relevant properties	Reference
pBAD-ABC	pBAD24 derivative containing the *E. coli tatABC* operon; Amp^r	Bolhuis *et al.*, 2001
pBAdCd	pBAD24 derivative containing the *B. subtilis tatAdCd* operon; Amp^r	Barnett *et al.*, 2008
pBAyCy	pBAD24 derivative containing the *B. subtilis tatAyCy* operon with 3' fusion to strep-II tag; Amp^r	This study
pBAyCys	pBAD24 derivative containing the *B. subtilis tatAyCy* operon with 3' fusion to strep-II tag; Amp^r	This study
pBAD-DmsA-GFP	pBAD24 derivative containing DmsA-GFP Amp^r	Ray *et al.*, 2003
pBAD-DmsA-GFP L19A	pBAD24 derivative containing DmsA-GFP Amp^r	Mendel *et al.*, 2008
pBAD-DmsA-GFP L19D	pBAD24 derivative containing DmsA-GFP Amp^r	Mendel *et al.*, 2008
pBAD-DmsA-GFP L19F	pBAD24 derivative containing DmsA-GFP Amp^r	Mendel *et al.*, 2008
pBAD-DmsA-GFP S15A	pBAD24 derivative containing DmsA-GFP Amp^r	Mendel *et al.*, 2008
pBAD-DmsA-GFP RK	pBAD24 derivative containing DmsA-GFP Amp^r	This study
pBAD-DmsA-GFP KR	pBAD24 derivative containing DmsA-GFP Amp^r	This study
pBAD-DmsA-GFP KK	pBAD24 derivative containing DmsA-GFP Amp^r	This study
pJDT1	pBAD24 derivative containing TorA-GFP Amp^r	Thomas *et al.*, 2001
pBAD-AmiA-GFP	pBAD24 derivative containing AmiA-GFP Amp^r	This study
pBAD-MdoD-GFP	pBAD24 derivative containing MdoD-GFP Amp^r	This study
pEXT-AdCd	pEXT22 derivative containing the *B. subtilis tatAdCd* operon; Kan^r	Barnett *et al.*, 2008
pEXT-AyCy	pEXT22 derivative containing the *B. subtilis tatAyCy* operon Kan^r	This study
Strains		
E. coli MC4100	F^- *Δlac*U169 *araD*139 *rpsL*150 *rel*A1 *ptsF rbs flbB*5301	Berthelmann *et al.*, 1998
MC4100 Δ*tatABCDE*	*tat* deletion strain	Wexler *et al.*, 2000
B. subtilis 168	*trpC2*	Kunst *et al.*, 1997

μM ammonium molybdate. Media were supplemented with ampicillin to a final concentration of 100 μg/ml, kanamycin to 50 μg/ml, arabinose to 0.5 mM and IPTG to 5 mM when required. *B. subtilis* was grown in TY (trypton/yeast extract) medium, consisting of Bactotryptone (1%; w/v), Bacto yeast extract (0.5%; w/v) and NaCl (1%; w/v), unless indicated otherwise. Media were supplemented with kanamycin (Km; 20 μg/ml), chloramphenicol (Cm; 5 μg/ml) and/or spectinomycin (Sp; 100 μg/ml).

DNA techniques.

For arabinose inducible overproduction of the *B. subtilis tatAyCy* operon with a C-terminal strep-II tag attached to the TatC component, plasmid pBAyCys was constructed as follows. The *tatAyCy* operon was amplified from *B. subtilis* 168 chromosomal DNA with primers RTEAyF (5'-CGC GTC TCG CAT GCC GAT CGG TCC TGG AAG CCT TGC TG-3') and JJystrep02 (5'-ATA TTC TAG ATT ATT TTT CAA ACT GTG GGT GCG ACC AAT TCG ATT GCC CAG AAG ACA CGT CCC G-3'). RTEAyF was designed as such that restriction of the generated *tatAyCy-strep* PCR-amplified fragment with dovetail enzyme *Esp*3I would create a *Nco*I overhang, to ensure direct cloning in the vector pBAD24. JJystrep02 was constructed as such that a C-terminal strep-II tag (underlined) would be directly attached to *tatCy* during the PCR amplification. pBAyCys was constructed by ligating an *Esp*3I- and *Xba*I cleaved PCR-amplified fragment of *tatAyCy* into *Nco*I- and *Xba*I cleaved pBAD24. For IPTG-inducible overproduction of *B. subtilis* TatAyCy, *tatAyCy-strep* was cut out of pBAyCys with *Nhe*I and *Xba*I and ligated into *Nhe*I/*Xba*I-cut pEXT22 to construct pEXT-AyCy.

For construction of pBAD-AmiA-GFP and MdoD-GFP the signal sequences for the two Tat substrates AmiA and MdoD were amplified by PCR from *E. coli* genomic DNA using the primers PCR_AmiA_EcoRI_for (GGC CGA ATT CAC CAT TAT GAG CAC TTT TA), and PCR_AmiA_EcoRI_rev (GGC CGA ATT CGC TGT GTC CGT TGC TGG TT) for AmiA, and PCR_MdoD_EcoRI_for (GGC CGA ATT CAC CAT TAT GGA TCG TAG AC) and PCR_MdoD_EcoRI rev (GGC CCA ATT CGT CAA AAC GCT GGG TTT GC) for MdoD. The PCR products were cut with *Eco*RI and then gel-purified. The expression vector pBAD24 containing *dmsA-GFP* was cut with *Eco*RI to release the DmsA signal sequence and then dephosphorylated after which the two PCR products *amiA* and *mdoD* were ligated into the vector (T4 Ligase NEBiolabs). The orientation of the two inserts was confirmed by sequencing.

Mutagenesis of the DmsA-GFP signal peptide was performed by site-directed

mutagenesis (Qiagen). Primers used were: KRDmsAF (CG GTA TTG GCT GCT GAG GTG AGT AAA CGT GGT TTG G) and KRDmsAR (CCA AAC CAC GTT TAC TCA CCT CAG CAG CCA ATA CCG) for KR mutation; RKDmsAF (GCT GCT GAG GTG AGT CGC AAA GGT TTG GTA AAA ACG) and RKDmsAR (CGT TTT TAC CAA ACC TTT GCG ACT CAC CTC AGC AGC) for RK mutation; and KRtoKKDmsAF (GCT GAG GTG AGT AAA AAG GGT TTG GTA AAA ACG ACA GCG) and KRtoKKDmsAR (CGC TGT CGT TTT TAC CAA ACC CTT TTT ACT CAC CTC AGC) for KK mutation.

SDS-PAGE and Western Blotting.

Proteins were separated using SDS polyacrylamide gel electrophoresis and immunoblotted using specific antibodies to TatAy and goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate. The *Strep*-tag II on TatCy was detected directly using a streptactin- HRP conjugate (Institut fur Bioanalytik). SufI, a Tat-dependent substrate of *E. coli*, was visualised using specific antibodies (kindly provided by T. Palmer). GFP was detected using a specific anti-GFP antibody (Promega) followed by goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate. An ECL detection kit (Amersham Pharmacia Biotech) was used to visualise the proteins.

TMAO Reductase activity and TatPre-GFP assays.

TMAO reductase activity assay was performed as described previously [33,36]. *E. coli* cells were grown anaerobically until mid-exponential growth phase prior to fractionation into periplasmic, cytoplasmic, and membrane fractions. Cell fractions were loaded and separated on a 10% native polyacrylamide gel that was subsequently assayed for TMAO reductase activity as described previously. Pre-GFP export assays: a construct comprising either the TorA [34], DmsA [27], MdoD, or AmiA signal peptide linked to green fluorescent protein (GFP) was expressed using the pBAD24 plasmid as previously described. For these experiments, TatAyCy was expressed from the compatible pEXT22 vector. Following expression from both plasmids, cell fractions were prepared as described above and immunoblotted using anti-GFP antibodies (Living Colors).

Expression and purification of the TatAyCy complex and TatAy complex.

E. coli Δ*tatABCDE* cells containing plasmid pBAyCys were grown aerobically to mid-exponential phase with induction of *tatAyCy* on plasmid pBAyCys using 0.5 mM arabinose. Cells were fractionated into membrane and cytosolic components as described previously, and the membranes were solubilised in 2% digitonin [33]. Solubilised membranes were incubated

with 2 μg/ml avidin to block any biotin-containing proteins before application to an equilibrated 4 ml Streptactin affinity column (Institut fur Bioanalytik). The column was washed with 10 column volumes of equilibration buffer containing Tris-HCl pH 8.0, 2% glycerol, 150 mM NaCl, and 0.1% digitonin. Bound protein was eluted from the column in 6 x 2.0 ml fractions using the same buffer as above but containing 3 mM desthiobiotin (Sigma). Elution fractions were pooled and diluted 50 fold in equilibration buffer to reduce the concentration of desthiobiotin in the eluted samples before application to a second 4 ml Streptactin affinity column. This time the column was washed with 5 column volumes of equilibration buffer and eluted in 6 x 2.0 ml fractions using the elution buffer described above. For gel filtration experiments affinity purified TatAyCy was concentrated to 250 l using Vivaspin-4 centrifugal concentrators (molecular weight cut off 10,000; Vivascience). The concentrated sample was loaded onto a Superose-6HR gel filtration column (Amersham Biosciences) and was eluted with the equilibration buffer described above [33].

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Chapter 6

High salinity growth conditions suppress the 'Sec avoidance' of potential Tat substrates in *Bacillus subtilis*

René van der Ploeg, Thijs R.H.M. Kouwen, Sjouke Piersma, James P. Barnett, Colin Robinson, and Jan Maarten van Dijl

ABSTRACT

The Gram-positive bacterium *Bacillus subtilis* contains two Tat translocases, which can facilitate transport of folded proteins across the plasma membrane. Previous research has shown that Tat-dependent protein secretion in *B. subtilis* is a highly selective process, and that heterologous proteins, such as the green fluorescent protein (GFP) are poor Tat substrates in this organism. Nevertheless, when expressed in *Escherichia coli*, both *B. subtilis* Tat translocases facilitated export of folded GFP provided with the twin-arginine (RR) signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins. Therefore, the present studies were aimed at determining whether the same RR-signal peptide-GFP hybrid precursors would also be exported Tat-dependently in *B. subtilis*. Unexpectedly however, these GFP fusion proteins were secreted Tat-independently in *B. subtilis*. At high salinity growth conditions, the Tat-independent secretion of GFP was significantly enhanced and this effect was strongest in strains lacking TatAyCy. This suggests that high environmental salinity has a negative influence on Sec avoidance by AmiA-GFP, DmsA-GFP and MdoD-GFP in *B. subtilis*. Over all, our observations indicate that as yet unidentified quality control mechanisms reject the investigated GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to their Sec-dependent secretion.

To be submitted for publication

INTRODUCTION

Protein secretion is an important feature for the survival and competitive success of bacterial cells in their natural habitats. The ability to secrete proteins is particularly well developed in the Gram-positive bacterium *Bacillus subtilis*, which is of interest both from applied and fundamental scientific points of view [1-4]. Combined genetic, proteomic and bioinformatic analyses have revealed that the vast majority of proteins secreted by *B. subtilis* leave the cytoplasm in an unfolded state via the general secretion (Sec) pathway [1]. Upon translocation these proteins fold into their active and protease-resistant conformation [5]. A limited number of proteins are secreted via the so-called twin-arginine (Tat) pathway which, in contrast to the Sec pathway, can facilitate the transport of fully folded proteins [6-10].

The proteins destined for export via the Sec or Tat pathways are synthesized with N-terminal signal peptides. These have a characteristic tripartite structure consisting of a positively charged N-terminal region, a hydrophobic H-region and a C-terminal region [3]. The C-region contains a signal peptidase cleavage site for signal peptide removal during or shortly after membrane translocation of the attached protein [11,12]. Although the signal peptides of Sec and Tat substrates are similar in structure, particular signal peptide features promote the specific targeting of proteins to the Tat pathway. These include a twin-arginine (RR) recognition motif in the N-region with the consensus sequence K/R-R-x-#-#, where # marks hydrophobic residues and x can be any residue [13-16]. This RR-motif is specifically recognized by the Tat translocase [17-19]. Additionally, RR-signal peptides are "unattractive" for the Sec machinery, because their H-region has a relatively low hydrophobicity, and because the C-region often (but not always) contains a positively charged residue that strongly promotes "Sec avoidance" [20] [14,21]. Importantly, the Sec incompatibility of Tat substrates is not only achieved through RR-signal peptide features, but also through their rapid or controlled folding in the cytoplasm prior to translocation [22-24]. In fact, some Tat-dependently exported proteins are subject to dedicated chaperone-mediated proofreading in the cytoplasm in order to prevent the initiation of their transport before folding or co-factor assembly have been completed [25,26]. As recently demonstrated by Matos *et al*., such quality control processes can also involve the Tat machinery itself [23,27].

B. subtilis contains two independently working Tat translocases named TatAyCy and TatAdCd, which are of the TatAC type that is commonly found in Gram-positive bacteria [28-30]. Unlike the TatABC type translocases that are present in Gram-

negative bacteria, these "minimal" TatAC translocases lack a TatB subunit [31-33]. In *B. subtilis*, the TatAyCy and TatAdCd translocases have distinct specificities for the Dyp-type peroxidase YwbN and the phosphodiesterase PhoD respectively, at least when the cells are grown in a standard LB medium [28-30]. Also, a hybrid precursor of the subtilisin AprE fused to the YwbN signal peptide was secreted in a TatAyCy-specific manner, suggesting a preferential interaction between the YwbN signal peptide and the TatAyCy translocase [34]. Nevertheless, the specificities of TatAyCy and TatAdCd overlap at least to some extent as was recently shown for Tat-dependent secretion of the esterase LipA in strains that hyper-produce this protein [35], and for Tat-dependent secretion of YwbN and the quality control protease WprA by cells grown in highly saline LB medium (chapter 4 of this thesis, unpublished observations). This view was further supported by the heterologous expression of TatAdCd or TatAyCy in *Escerichia coli* strains lacking their own TatABC translocase [32,33]. The latter studies revealed that both *B. subtilis* Tat translocases are able to translocate the green fluorescent protein (GFP) fused to the RR-signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins (Fig. 1). A specificity difference was, however, observed also in *E. coli* as the TMAO reductase (TorA) and a TorA-GFP fusion were transported by TatAdCd but not by TatAyCy [32,33]. An interesting conclusion from the heterologous Tat expression studies in *E. coli* was that both *B. subtilis* TatAC translocases were able to translocate active GFP when expressed in *E. coli*. By contrast, earlier experiments had indicated

AmiA MSTFKPLKTLTSRRQVLK*AGLAALTLSGMS***QAIAK**DELLKTSNGHS

DmsA MKTKIPDAVLAAEVSRRGLVK*TTAIGGLAMASSALTLPFS***RIAHAV**

MdoD MDRRRFIK*GSMAMAAVCGTSGIASLFS***QAAFAA**DSDIADGQTQRFD

LipA MKFVKRR*IIALVTILMLSVTSLFAL***QPSAKAA**EHNPVVMVHGIGGASF

YwbN MSDEQKKPEQIHRRDILK*WGAMAGAAVAIGASGLGGLAP***LVQTAA**K

Figure. 1. Signal peptide sequences. The amino acid sequences of the RR-signal peptides of AmiA, DmsA and MdoD of *E. coli*, and LipA and YwbN of *B. subtilis* are shown. Twin-arginine motifs are underlined, hydrophobic H-regions are printed in italics, and the C-regions are marked in bold with residues flanking the signal peptidase cleavage sites underlined.

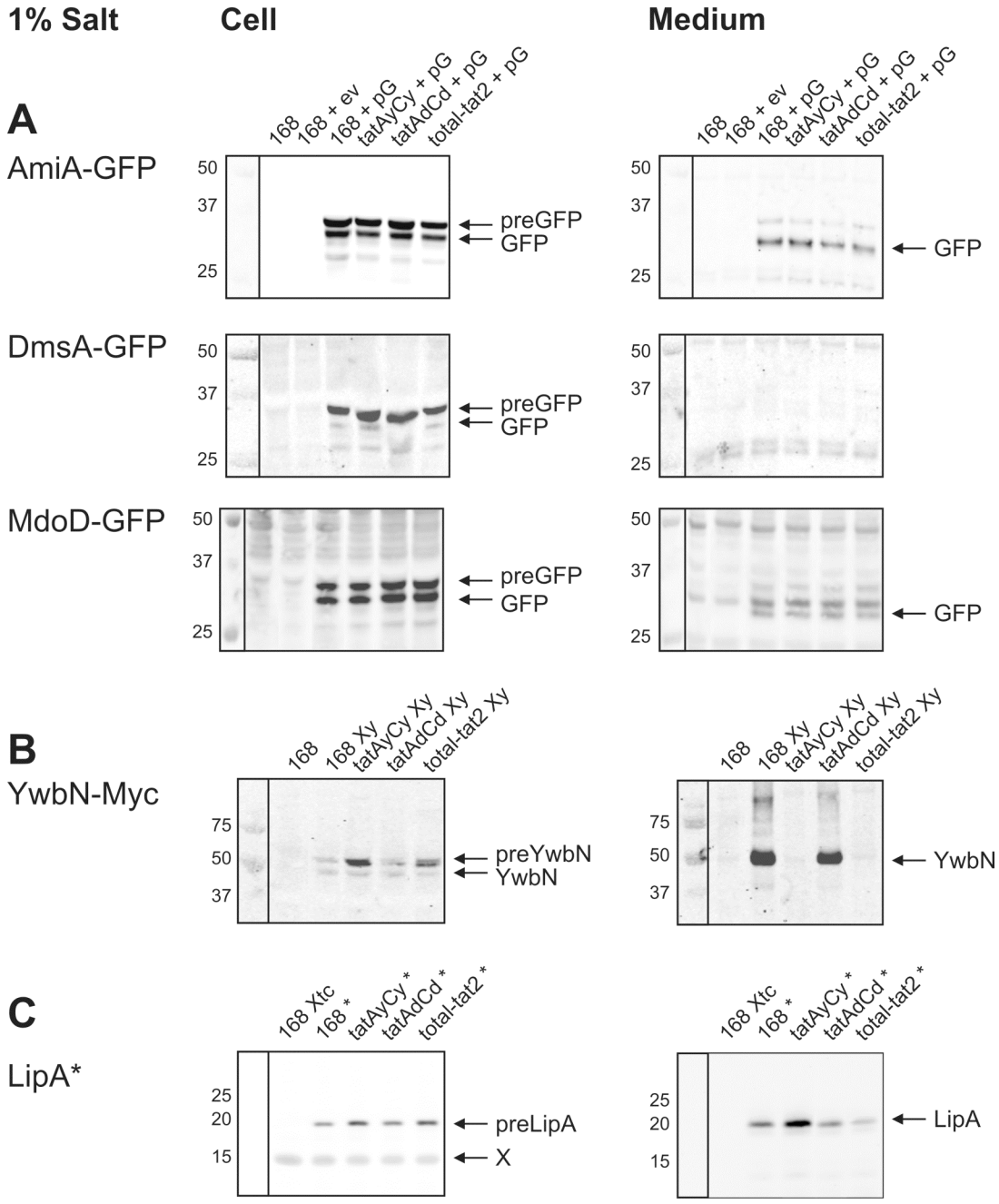
that this was not possible in *B. subtilis* [34,36]. Therefore, the aim of the present studies was to assess whether the same RR-signal peptide-GFP hybrid precursors that were Tat-dependently translocated in *E. coli* would also lead to Tat-dependent GFP secretion in *B. subtilis*. Briefly, the results show that this is not the case. Instead, Tat-independent GFP secretion was observed, which was most pronounced when the cells were grown in LB medium of high salinity. Taken together, our findings indicate that as yet unidentified quality control mechanisms reject the GFP fusion proteins for translocation by the *B. subtilis* Tat machinery. Furthermore, the Sec avoidance of all three hybrid GFP precursors seems to be suppressed when cells are grown in medium with 6% salt.

RESULTS

The AmiA and MdoD RR-signal peptides mediate Tat-independent GFP secretion in *B. subtilis*.

When heterologously expressed in *E. coli*, the TatAdCd and TatAyCy translocases can transport the AmiA-GFP, DmsA-GFP and MdoD-GFP precursors across the inner membrane, leading to an accumulation of active GFP in the periplasm [32,33]. To assess whether the very same RR-signal peptide-GFP precursors would also be exported Tat-dependently in *B. subtilis*, we expressed them in *B. subtilis* 168 and corresponding *tat* mutant strains. For this purpose, the respective hybrid genes were provided with the ribosome-binding

Figure 2. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in standard LB medium. (A) Cell and growth medium fractions of *B. subtilis* strains producing AmiA-GFP, DmsA-GFP or MdoD-GFP were separated by centrifugation and used for SDS-PAGE and Western blotting with specific antibodies. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168 were grown for 7 hours in LB medium, supplemented with 1% NaCl . Protein loading was corrected for OD_{600}. "pG", cells harbouring pHB-AmiA-GFP, pHB-DmsA-GFP or pHB-MdoD-GFP; "ev", cells harbouring the empty vector pHB201. **(B)** Cell and growth medium fractions of *B. subtilis* strains producing YwbN-Myc were prepared for SDS-PAGE and Western blotting with specific antibodies as indicated for panel A. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168 contained the X*ywbN* cassette in *amyE*. "Xy", cells containing the X*ywbN* cassette. **(C)** Cell and growth medium fractions of *B. subtilis* strains hyper-producing LipA were prepared for SDS-PAGE and Western blotting with specific antibodies as indicated for panel A. For this purpose, we used the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant XdsbA* strains or the parental strain 168 XdsbA*, all of which contain an unidentified mutation that leads to LipA hyper-production (marked *). The parental strain 168 Xtc, which lacks the mutation for LipA hyper-production was used as a control. The positions of precursor and mature forms of GFP, YwbN-Myc and LipA are marked with arrows. Positions of Mw markers are indicated on the left (kDa). The X marks an unidentified protein, that cross reacts with the LipA antibody.



site *plus* start codon of the *B. subtilis mntA* gene, which are very well suitable for heterologous protein expression in *B. subtilis* [37]. The resulting constructs were then constitutively expressed at relatively low levels from the *E. coli* - *B. subtilis* shuttle vector pHB201. As shown in Figure 2A (left panels), all three precursors were synthesized in *B. subtilis* cells grown overnight in standard LB medium with 1% NaCl. However, only in the case of AmiA-GFP and MdoD-GFP effective conversion to the mature form and release of this mature form into the growth medium were

observed (Fig. 2A, left and right panels). The strains producing AmiA-GFP secreted relatively higher amounts of mature GFP into the medium than strains producing MdoD-GFP. Notably, the secretion of mature-sized GFP by strains producing AmiA-GFP was not influenced by the absence of *tatAyCy*, *tatAdCd* or even all *tat* genes, and the same was true for strains producing MdoD-GFP albeit that GFP was secreted at lower levels (Fig. 2A). By contrast, under these conditions the control protein YwbN-Myc was secreted in a strictly TatAyCy-dependent manner as evidenced by the fact that it was secreted only by the parental strain 168 and the *tatAdCd* mutant, but not by the *tatAyCy* or total-*tat2* mutants (Fig. 2B). Likewise, the hyperproduced esterase LipA was secreted at strongly reduced levels by the total-*tat2* mutant (Fig. 2C), which is in agreement with our previous findings [35]. While the production of AmiA-GFP and MdoD-GFP resulted in Tat-independent GFP secretion, no secretion of GFP was detectable for wild-type or *tat* mutant strains producing the DmsA-GFP precursor (Fig. 2A). Consistent with this observation, barely any mature-sized GFP was detectable in cells producing DmsA-GFP. This suggests that the DmsA-GFP precursor is neither an acceptable substrate for the two TatAC translocases nor the Sec translocase produced in *B. subtilis* cells grown in standard LB medium.

High salinity growth conditions result in elevated levels of Tat-independent GFP secretion.

We have previously shown that the specificity of Tat-dependent protein transport in *B. subtilis* is influenced by the salinity of the growth medium (chapter 4). This is most clearly evidenced by the finding that the YwbN protein requires both the TatAdCd and TatAyCy translocases for efficient secretion when cells are grown in LB medium with 6% salt (Fig. 3B). Additionally, some YwbN is secreted completely Tat-independently under these conditions. To investigate whether the secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP might be influenced by a growth medium with high salinity, cells producing these hybrid precursors were grown in LB medium with 6% NaCl. As shown by Western blotting of cellular and growth medium samples, the increased salt concentration in the medium resulted in a drastically improved signal peptidase processing and secretion of DmsA-GFP since mature-sized GFP was now detectable in cellular and growth medium fractions (Fig. 3A). The highest levels of secreted GFP were observed for the *tatAyCy* and total-*tat* mutant strains, suggesting that the TatAyCy translocase interferes with the Tat-independent translocation of DmsA-GFP during growth in LB medium with 6% salt. Consistent with these findings, the high salinity growth conditions also had

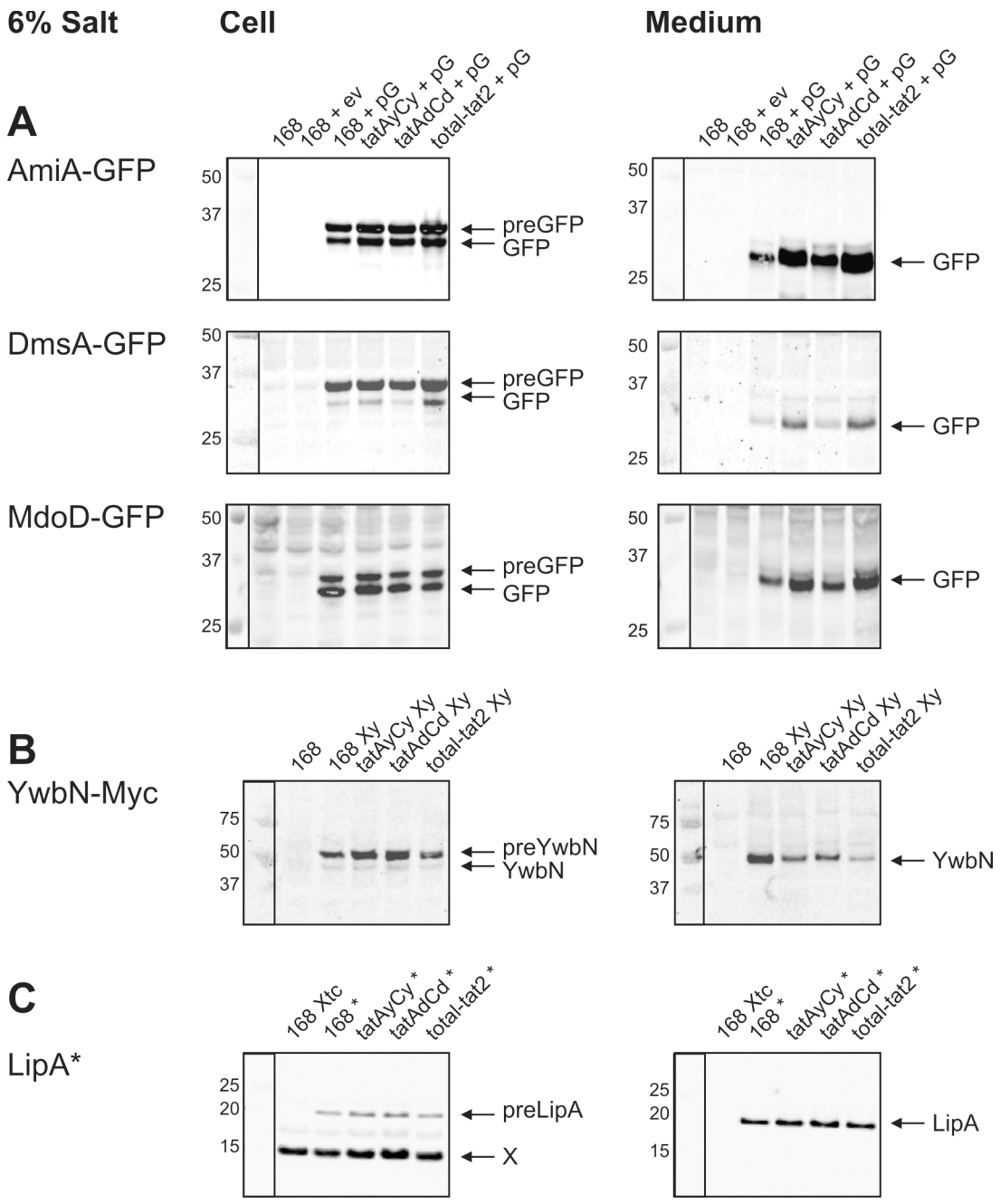


Figure 3. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in LB medium with 6% NaCl. Cell and growth medium fractions of *B. subtilis* strains producing AmiA-GFP, DmsA-GFP or MdoD-GFP **(A)**, producing YwbN-Myc **(B)**, or hyper-producing LipA **(C)** were separated by centrifugation and used for SDS-PAGE and Western blotting with specific antibodies. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168 were grown for 7 hours in LB medium, supplemented with 6% NaCl. Protein loading was corrected for OD_{600}. Lanes are labelled as in Figure 2, and the positions of precursor and mature forms of GFP, YwbN-Myc and LipA are marked with arrows. Positions of Mw markers are indicated on the left (kDa).

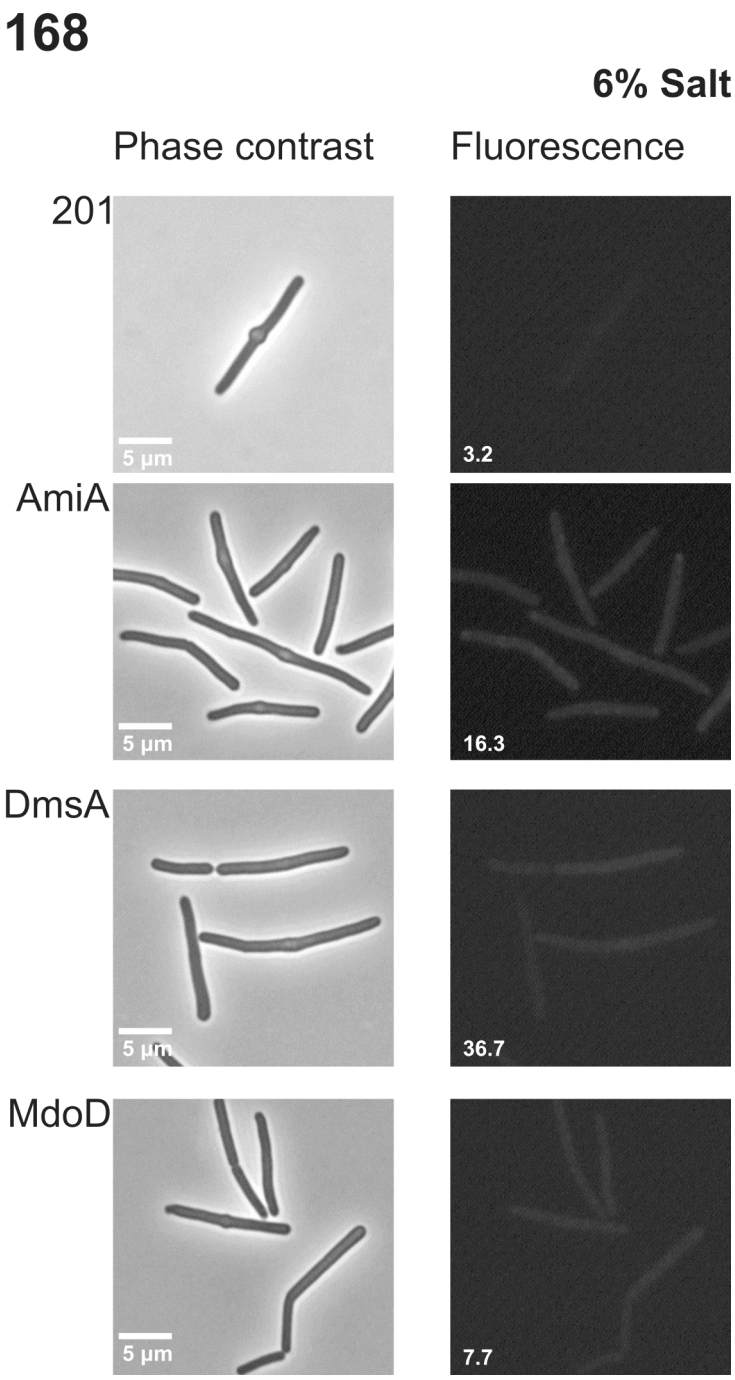


Figure 4. Fluorescence microscopic analysis of GFP production. Cells of *B. subtilis* 168 producing AmiA-GFP (AmiA), DmsA-GFP (DmsA), MdoD-GFP (MdoD) or no GFP (strain containing the empty vector pHB201) were grown in LB medium with 6% NaCl for 7 h. Cells were spotted onto M9 agarose slides with 6% NaCl and analyzed by phase contrast and fluorescence microscopy. The cellular fluorescence values indicated in the fluorescence panels were determined as arbitrary grey scale units of the cells and have been corrected for average background fluorescence.

a clearly stimulating effect on the secretion of mature GFP by cells producing AmiA-GFP or MdoD-GFP. Also here, the highest levels of mature GFP were secreted by the *tatAyCy* and total-*tat* mutant strains producing AmiA-GFP or MdoD-GFP. Finally, high salinity growth conditions were found to completely abolish the Tat-dependent secretion of hyper-produced LipA (Fig. 3C).

Taken together, these observations show that the Tat-independent secretion of GFP, LipA and YwbN is strongly stimulated when cells are grown in LB medium with 6% NaCl. This implies that such high salinity growth conditions result in an, at least partially, suppressed Sec avoidance of the respective precursors proteins. Since both Tat-dependent protein translocation and Sec avoidance are not only determined by features of the signal peptide, but also by the folding state of the respective precursor protein, we used fluorescence microscopy to determine whether folded and active GFP is detectable in cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP. Indeed Figure 4 shows that at least some of the GFP within cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP is active. Nevertheless, little if any of this folded GFP seems to be secreted by the Tat translocases of the respective cells.

DISCUSSION

The present studies were aimed at investigating the possible Tat-dependent secretion in *B. subtilis* of hybrid GFP precursor proteins that contain the RR-signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins. While these precursors were previously shown to be transported to the periplasm of *E. coli* by the heterologously expressed TatAdCd or TatAyCy translocases of *B. subtilis* [32,33], we now show that these precursors are not accepted by the *B. subtilis* TatAC translocases when expressed in *B. subtilis*. Instead, Tat-independent secretion of GFP was observed in strains producing the AmiA-GFP or MdoD-GFP precursors under standard growth conditions (*i.e.* LB medium with 1% NaCl), and this Tat-independent secretion was significantly enhanced when the strains were grown in LB medium with 6% NaCl. While cells expressing the DmsA-GFP precursor under standard growth conditions did not secrete GFP, these cells did secrete matured GFP Tat-independently when grown in LB with 6% NaCl. Under these high salinity growth conditions, we even observed Tat-independent secretion of the known *B. subtilis* Tat substrate YwbN, and the secretion of hyperproduced LipA, which depends to a large extent on Tat, was completely Tat-independent under these

conditions. These findings imply that the Sec avoidance of *B. subtilis* RR-precursor proteins under standard growth conditions is suppressed under high salinity growth conditions.

Previous studies have indicated that the Tat pathway in *B. subtilis* is able to facilitate the secretion of GFP, albeit in an inactive state (Meissner *et al*. 2007). It is therefore not clear why the *B. subtilis* TatAC translocases do not facilitate the secretion of mature GFP when the AmiA-GFP, DmsA-GFP or MdoD-GFP precursors are produced in *B. subtilis*. At least two possible reasons for this finding are conceivable. Firstly, the respective RR-signal peptides may not be presented to the TatAC translocases in the right way. This would then expose these signal peptides to the Sec machinery of *B. subtilis*, resulting in Tat-independent GFP secretion via the Sec pathway. Consistent with this idea, the RR-motifs in the AmiA, DmsA and MdoD signal peptides do not show a perfect match with the consensus RR-motif S/T-R-R-x-F-L-K (Fig. 1). Nevertheless, at least under high salinity growth conditions, the RR-signal peptides of AmiA, DmsA and MdoD seem to be recognized by TatAyCy as was evidenced by the observation that Tat-independent GFP secretion was enhanced in *B. subtilis* strains lacking *tatAyCy*. Secondly, the GFP attached to the AmiA, DmsA or MdoD signal peptides may not fold rapidly enough in *B. subtilis* to allow Tat-dependent translocation of AmiA-GFP, DmsA-GFP or MdoD-GFP. As shown by fluorescence microscopy, the cells expressing these precursor proteins did contain active GFP, but this does of course not rule out the possibility that substantial amounts of GFP do not fold fast enough to avoid their translocation via Sec. In this case, a so far unidentified quality control step in the *B. subtilis* Tat pathway would reject the three precursors prior to translocation. Such a quality control step might involve particular proteinaceous determinants, such as the *B. subtilis* TatD homologue YabD. Notably, Tullman-Ercek *et al*. [21] reported that the signal peptides of AmiA, DmsA and MdoD can direct attached proteins, such as GFP, the alkaline phosphatase PhoA and the maltose-binding protein MBP to both the Sec and Tat pathways of *E. coli*. The Tat-specificity of the AmiA and MdoD signal peptides was found to be especially low when fused to the alkaline phosphatase PhoA, which is a regular Sec substrate [21]. However, the Tat-independent export of GFP fused to the AmiA and MdoD signal peptides was also substantial (about 25-30%), which is consistent with our present finding that these hybrid precursors are Tat-independently exported in *B. subtilis*. Furthermore, the export of DmsA-GFP in *E. coli*, as reported by Tullman-Ercek *et al*. was only to less than 10% Tat-independent,

which is line with our present observations that synthesis of this precursor does not lead to detectable levels of Tat-independent secretion of GFP. The observed strong Sec avoidance of DmsA-GFP is consistent with the presence of two positively charged residues in the C-region of the DmsA signal peptide (*i.e.* Arg and His; Fig. 1). Such positively charged residues with a possible role in Sec avoidance are absent from the AmiA and MdoD signal peptides.

Interestingly, an increased salinity of the growth medium seems to result in a suppression of Sec avoidance, not only by the AmiA-GFP, DmsA-GFP and MdoD-GFP precursurs, but also by authentic Tat-dependently secreted proteins such as YwbN and LipA. It is presently not clear why this happens, but the finding suggests that electrostatic interactions and/or a salt-sensitive factor are involved in Sec avoidance. A possible involvement of electrostatic interactions in Sec avoidance would be in line with the finding that positively charged residues in the C-region of the signal peptide facilitate Sec avoidance. However, high salinity of the growth medium may also slow down the folding of precursor proteins, which would then make these proteins more attractive for the Sec translocase. One additional Sec-avoidance determinant seems to be the TatAyCy translocase itself, since the absence of this translocase resulted in increased levels of GFP secretion under high salinity growth conditions. It thus seems that TatAyCy can be directly involved in Sec avoidance, possibly by targeting unfolded GFP precursors for degradation, or by redirecting them into the cytoplasm where they fold into a Sec incompatible state. This finding is thus reminiscent of the recently reported involvement of the *E. coli* Tat translocase together with TatD in the rejection of malfoded normally Tat-dependent precursor proteins [23,27].

Notably, in *B. subtilis* an increased TatAdCd-dependent secretion in the absence of TatAyCy has previously been shown for the phosphodiestase PhoD, hyper-produced LipA [28,29] and the WprA protease (chapter 4). This supports the view that interactions of certain precursor proteins with TatAyCy may lead to the rejection of these precursors for translocation via Tat in *B. subtilis*.

In conclusion, the present results indicate that as yet unidentified quality control mechanisms reject the AmiA-GFP, DmsA-GFP and MdoD-GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to their Sec-dependent secretion. At least the Sec avoidance of all three hybrid GFP precursors seems to be overruled when cells are grown in LB medium with 6% salt. Furthermore, at least under these high salinity growth conditions, the TatAyCy translocase seems to be a determinant in

Sec avoidance. Most likely, the identification and subsequent elimination or modulation of the quality control systems that determine GFP secretion will be key to unlocking the *B. subtilis* Tat pathway for the production of heterologous proteins.

MATERIALS AND METHODS

Plasmids, bacterial strains, media and growth conditions.

The plasmids and bacterial strains used in this study are listed in Table I. Strains were grown with agitation at 37ºC in either Luria Bertani-Miller (LB) medium, or Paris minimal (PM) medium. LB medium consisted of 1% tryptone, 0.5% yeast extract and 1% or 6% NaCl, pH 7.4. Notably, LB with 1% NaCl is the standard LB medium that has been used in all our previous studies. PM consisted

Table I Strains and Plasmids

Plasmids	Relevant properties	Reference
pHB201	*B. subtilis-E. coli* expression vector; ori-pBR322; ori-pTA1060; *cat*86::*lacZa*; Cm^R; Em^R	Bron *et al*., 1998
pHB-AmiA-GFP	pHB201 vector carrying the *amiA-gfp* hybrid *gene*; Cm^R; Em^R	This study
pHB-DmsA-GFP	pHB201 vector carrying the *dmsA-gfp* hybrid *gene*; Cm^R; Em^R	This study
pHB-MdoD-GFP	pHB201 vector carrying the *mdoD-gfp* hybrid *gene*; Cm^R; Em^R	This study
Strains		
E. coli		
DH5α	*supE*44; *hsdR*17; recA1; gyrA96; thi-1; relA1	Sambrook *et al.,* 1989
B. subtilis		
168	*trpC2*	Antelmann *et al*., 2003
tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; Sp^R	Jongbloed *et al.*, 2002
tatAdCd	*trpC2*; *tatAd-tatCd*::Km; Km^R	Jongbloed *et al.*, 2004
total-tat_2	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; *tatAc*::Em; Em^R	Jongbloed *et al.*, 2004
168 *XywbN*	*trpC2*; *amyE*::xylA-*ywbN-myc*; Cm^R	Jongbloed *et al.*, 2004
tatAyCy XywbN	*trpC2*; *tatAy-tatCy*::Sp; Sp^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al.*, 2004
tatAdCd XywbN	*trpC2*; *tatAd-tatCd*::Km; Km^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al.*, 2004
total-tat_2 *XywbN*	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; *tatAc*::Em; Em^R; *amyE*::*xylA-ywbN-myc*; Cm^R	Jongbloed *et al.*, 2004

Table I continued; Strains and Plasmids

Strains	Relevant properties	Reference
168 pHB201	*trpC2*; pHB201; Em^R; Cm^R	This study
168 pHB-AmiA-GFP	*trpC2*; pHB-AmiA-GFP; Em^R; Cm^R	This study
tatAyCy pHB-AmiA-GFP	*trpC2*; *tatAy-tatCy*::Sp; pHB-AmiA-GFP; Sp^R; Em^R; Cm^R	This study
tatAdCd pHB-AmiA-GFP	*trpC2*; *tatAd-tatCd*::Km; pHB-AmiA-GFP; Km^R; Em^R; Cm^R	This study
total-tat_2 pHB-AmiA-GFP	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; *tatAc*::Em; pHB-AmiA-GFP Km^R; Sp^R; Em^R; Cm^R	This study
168 pHB-DmsA-GFP	*trpC2*; pHB-DmsA-GFP; Em^R; Cm^R	This study
tatAyCy pHB-DmsA-GFP	*trpC2*; *tatAy-tatCy*::Sp; pHB-DmsA-GFP; Sp^R; Em^R; Cm^R	This study
tatAdCd pHB-DmsA-GFP	trpiC2; *tatAd-tatCd*::Km; pHB-DmsA-GFP; Km^R; Em^R; Cm^R	This study
total-tat_2 pHB-DmsA-GFP	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; *tatAc*::Em; pHB-DmsA-GFP Km^R; Sp^R; Em^R; Cm^R	This study
168 pHB-MdoD-GFP	*trpC2*; pHB-MdoD-GFP; Em^R; Cm^R	This study
tatAyCy pHB-MdoD-GFP	*trpC2*; *tatAy-tatCy*::Sp; pHB-MdoD-GFP; Sp^R; Em^R; Cm^R	This study
tatAdCd pHB-MdoD-GFP	*trpC2*; *tatAd-tatCd*::Km; pHB-MdoD-GFP; Km^R; Em^R; Cm^R	This study
total-tat_2 pHB-MdoD-GFP	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; *tatAc*::Em; pHB-MdoD-GFP Km^R; Sp^R; Em^R; Cm^R	This study
168 Xtc	*trpC2*; *amyE*::XTC; integrated "empty" XTC cassette; Tc^R [35]	Kouwen *et al.*, 2009
168 XdsbA*	*trpC2*; *amyE*::XTC*dsbA*; integrated XTC cassette carrying *dsbA* of *S. aureus*; Tc^R [35]	Kouwen *et al.*, 2009
tatAyCy XdsbA*	*trpC2*; *tatAy-tatCy*::Sp; *amyE*::XTC*dsbA*; integrated XTC cassette carrying *dsbA* of *S. aureus*; Tc^R [35]	Kouwen *et al.*, 2009
tatAdCd XdsbA*	*trpC2*; *tatAd-tatCd*::Km; *amyE*::XTC*dsbA*; integrated XTC cassette carrying *dsbA* of *S. aureus*; Tc^R [35]	Kouwen *et al.*, 2009
tatAyCy tatAdCd XdsbA*	*trpC2*; *tatAd-tatCd*::Cm; *tatAy-tatCy*::Sp; *amyE*::XTC*dsbA*; integrated XTC cassette carrying *dsbA* of *S. aureus*; Tc^R [35]	Kouwen *et al.*, 2009

of 10.7 mg/ml K2HPO4, 6 mg/ml KH2PO, 1 mg/ml trisodium citrate, 0.02 mg/ml MgSO4, 1% glucose, 0.1% casamino acids (Difco), 20 mg/ml L-tryptophan, 2.2 mg/ml ferric ammonium citrate and 20 mM potassium glutamate. When required, media for *E. coli* were supplemented with erythromycin (Em; 100 μg/ml), kanamycin (Km; 20 μg/ml), chloramphenicol (Cm; 5 μg/ml), or spectinomycin (Sp; 100 μg/ml); media for *B. subtilis* were supplemented with Em (2 μg/ml), Km (20 μg/ml), Cm (5 μg/ml) or Sp (100 μg/ml).

DNA techniques.

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as previously described [38]. *B. subtilis* was transformed as described by Kunst and Rapoport [40]. PCR was carried out with the Pwo DNA polymerase. PCR products were purified using the PCR purification kit from Roche. Restriction enzymes were obtained from New England Biolabs. Plasmid DNA from *E. coli* was isolated using the alkaline lysis method [38], or the Invisorb®Plasmid Isolation Kit (Invitek). All constructs were checked by sequencing (serviceXS, Leiden the Netherlands).

To construct the plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and pHB-MdoD-GFP, the *amiA-gfp*, *dmsA-gfp* and *mdoD-gfp* hybrid genes were PCR-amplified from the respective pBAD24-based plasmids carrying these genes [32] (Table I). The 5' primers used for PCR contained the *mntA* ribosome-binding site and start codon, as well as a *Spe*I restriction site, and the 3' primer contained a *Bam*HI restriction site (Table II). The resulting PCR products were cleaved with *Spe*I and *Bam*HI, and ligated to *Spe*I-*Bam*HI-cleaved pHB201. Ligation mixtures were used to transform *E. coli*, resulting in the identification of plasmids pHB-AmiA-GFP, pHB-DmsA- GFP and pHB-MdoD-GFP. Next, these plasmids were used to transform the *B. subtilis* strains 168, *tatAyCy*, *tatAdCd* and total-tat_2.

SDS-PAGE and Western blotting.

Cellular or secreted proteins were separated by SDS-PAGE using pre-cast Bis-Tris NuPAGE gels (Invitrogen). The presence of GFP, YwbN or LipA in cellular or growth medium fractions was detected by Western blotting. For this purpose, proteins separated by SDS-PAGE were semi-dry blotted (75 min at 1 mA / Cm^2) onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). Subsequently, GFP was detected with monoclonal antibodies (Clontech), YwbN-Myc was detected with monoclonal antibodies against the Myc-tag attached to this protein (Gentaur), and LipA was detected with specific polyclonal antibodies raised in rabbits. Visualisation of bound antibodies was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm.

Fluorescence microscopy.

Cells were grown in LB without NaCl or supplemented with 1 or 6% NaCl. After 7 hours of growth the optical density at 600 nm (OD_{600}) was measured. Cells were then incubated for another 20 minutes and spotted on M9 agarose slides containing the appropriate salt concentrations. These slides

were prepared by transfer of M9 agarose medium into a 65 µl Frame-Seal Slide Chamber (SLF-0601, Bio-Rad). Fluorescence microscopy was performed with a Leica DM5500 B microscope. Fluorescence images were recorded using a Leica EL6000 lamp with the intensity set to 55%. The exposure time was 256 ms. Quantification of GFP fluorescence was done using the ImageJ software package (http://rsbweb.nih.gov/ij/). Cellular fluorescence values were measured in grey scale values. Background fluorescence was calculated by averaging the grey scale values of the area outside the cells. Finally the background fluorescence was subtracted from the cellular fluorescence.

Table II Primers

Primer	Sequence	Remarks
RBS-MntA-AmiA-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGAGCACTTTTAAACCACTA*	*Spe*I, RBS *mntA* start *amiA*
RBS-MntA-DmsA-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGAAAACGAAAATCCCTGAT*	*Spe*I, RBS *mntA* start *dmsA*
SpeI-MntA-MdoD-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGGATCGTAGACGATTTATT*	*Spe*I, RBS *mntA* start *mdoD*
GFP-Rev-BamHI	CCCCCGGATCCTTATTTGTATAGTTCATCCATGC	*Bam*HI, end *gfp*

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Chapter 7

Intrinsic salt-sensitivity of minimal TatAC translocases

René van der Ploeg, James P. Barnett, Nishi Vasisht, Dierk Poether, Colin Robinson, Jan Maarten van Dijl

ABSTRACT

Bacterial Twin-arginine translocation (Tat) pathways have evolved to facilitate transport of folded proteins across the plasma membrane. Gram-negative bacteria have a TatABC translocase composed of three subunits named TatA, TatB and TatC. In contrast, the Tat translocases of most Gram-positive bacteria consist of only TatA and TatC subunits. In these minimal TatAC translocases, a bifunctional TatA subunit fulfils the roles of both TatA and TatB. Here we have studied the function of the bifunctional TatAy subunit from *Bacillus subtilis* by site-specific mutagenesis of conserved residues. This yielded a set of engineered TatAy proteins with mutations in the cytoplasmic hinge and amphipathic helix regions, which were inactive for protein translocation under standard growth conditions for *B. subtilis*, and when heterologously expressed in *Escherichia coli*. Nevertheless, these mutant TatAy proteins did engage in TatAy and TatAyCy complexes, and facilitated membrane association of twin-arginine precursor proteins in *E. coli*. Interestingly, the respective mutant TatAyCy translocases were found to be conditionally salt-sensitive in *B. subtilis*. Similarly, the TatAC translocases of *Bacillus cereus* and *Staphylococcus aureus* were found to be intrinsically salt-sensitive when expressed in *B. subtilis*. Taken together, our present observations suggest that salt-sensitive electrostatic interactions play important roles in the preprotein translocation activity of TatAC type translocases from Gram-positive bacteria.

To be submitted for publication

INTRODUCTION

Bacterial, archaeal and thylakoidal twin-arginine translocation (Tat) pathways facilitate the passage of fully folded substrate proteins across biological membranes without compromising the respective transmembrane ion gradients [1-8]. There are two key prerequisites for the transport of substrate proteins via Tat. Firstly, these proteins need to have an N-terminal signal peptide with a characteristic twin-arginine (RR-) motif (K/R-R-X-F-L-K; [9-15]). The RR-signal peptide facilitates precursor targeting to the Tat translocase and initiation of the translocation process. In fact, the Tat pathway was named after the two adjacent (twin) arginine residues in the RR-motif. Secondly, the substrate protein, in general, needs to be presented to the Tat translocase in a folded state [16-18].

In bacteria, two types of Tat translocases have been identified. Gram-negative bacteria and a few Gram-positive bacteria, such as streptomycetes, have TatABC translocases [19-22]. These translocases are composed of three subunits – TatA, TatB and TatC – that are of critical importance for translocation activity. TatABC-type translocases are also present in the chloroplast thylakoidal membrane [23-25]. The vast majority of Gram-positive bacteria contain TatAC translocases that are composed of TatA and TatC subunits only [26-28]. In this case, the TatA subunit is bifunctional, fulfilling the roles of both TatA and TatB [29-31]. Although the precise modes of action of TatABC and TatAC translocases have not yet been fully elucidated, the consensus mechanistic model envisages that the RR-motif in signal peptides is recognized by a TatBC receptor complex in Gram-negative bacteria, or a TatAC receptor complex in Gram-positive bacteria complex [30,32-34]. The TatBC- or TatAC-precursor complexes would subsequently associate with TatA complexes that co-exist as separate entities in the membrane. This in turn would lead to the formation of a flexible channel composed of multiple TatA subunits, facilitating membrane passage of substrate proteins that can range in diameter from ~20 to ~70 Å [34-37]. The proton-motive force is believed to strengthen the signal peptide-receptor complex interaction and to drive protein translocation across the membrane [34,35] [38,39].

In certain bacteria, such as the Gram-positive bacterium *Bacillus subtilis*, multiple paralogues of TatA or TatC are present. Specifically, *B. subtilis* contains two TatAC systems, named TatAdCd and TatAyCy, which are encoded by the *tatAd-tatCd* and *tatAy-tatCy* operons [26,29]. Furthermore, the *tatAc* gene encodes an "orphan" TatA subunit of unknown function. The TatAdCd and TatAyCy translocases have different but overlapping

specificities, and their usage is strongly dependent on growth conditions (chapter 4). Under conditions of phosphate starvation, the TatAdCd translocase is responsible for secretion of the phosphodiesterase PhoD, while TatAyCy is dispensable for this process [26,40]. Conversely, when cells are cultivated in LB medium with no or 1% added NaCl, the TatAyCy translocase secretes the Dyp-type peroxidase YwbN without any detectable involvement of TatAdCd [29] (chapter 4). However, compared to cells grown in the standard LB medium with 1% NaCl, the overall Tat pathway usage changes when cells are grown in LB medium without NaCl, or with 6% NaCl. Thus, the cell wall hydrolase LytD is secreted TatAyCy-dependently when cells are grown in LB medium without added salt. Under high salinity growth conditions, TatAyCy and TatAdCd are both required for YwbN secretion. Also, the quality control protease WprA is secreted in a TatAyCy-TatAdCd-dependent manner by cells growing in LB with 6% NaCl (chapter 4). Another example of conditional Tat pathway usage in *B. subtilis* came from studies showing that the esterase LipA is secreted in a TatAyCy-TatAdCd-dependent manner by cells hyper-producing this protein [41]. Recently, the overlapping specificity of the TatAdCd and TatAyCy translocases was confirmed also by their heterologous expression in *Escherichia coli* [31]. While several studies have addressed structure-function relationships in the TatA and TatB subunits of the TatABC translocase from *E. coli* [42-48], such studies have thus far been lacking for bifunctional TatA subunits that are part of the TatAC translocases of Gram-positive bacteria. This triggered us to assess the functions of conserved residues in the *B. subtilis* TatAy protein by site-specific mutagenesis. To monitor the activity of translocases with mutant TatAy subunits, we used the secreted YwbN protein as a reporter. By doing so, we took advantage of the high specificity of the TatAyCy translocase for YwbN when cells are grown in LB without NaCl or with 1% NaCl. An additional advantage of using TatAy for mutagenesis studies is that TatAyCy-dependent YwbN secretion is required for achieving wild-type growth rates and stationary phase survival of cells grown in LB without NaCl (chapter 4). This relates to a critical role of YwbN in the acquisition of iron under these conditions. The present studies identify several residues that are critical for TatAy activity when cells are grown in LB medium with 1% NaCl. The inactive mutant TatAy proteins are all capable of engaging in TatAy and TatAyCy complexes, and they facilitate membrane association of a hybrid RR-precursor protein. This implies, that the respective mutations interfere with translocation activity of TatAyCy rather than complex assembly.

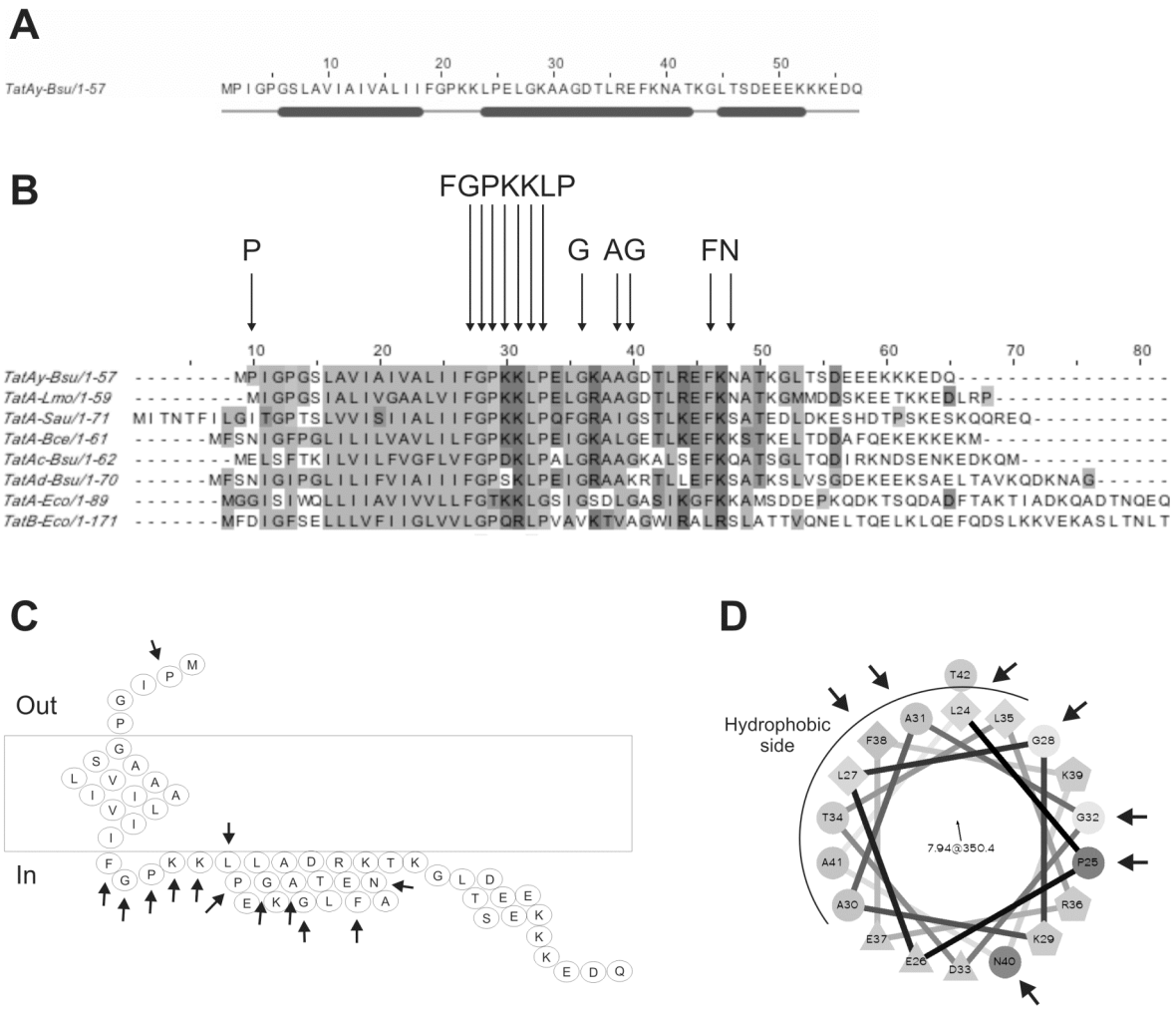


Figure 1. The *B. subtilis* TatAy protein. (A) Secondary structure prediction of TatAy indicating the location of alpha-helical regions. **(B)** Sequence alignment of TatAy with TatA proteins from *B. subtilis* (*Bsu*), *L. monocytogenes* (*Lmo*), *S. aureus* (*Sau*) and *B. cereus* (*Bce*) with TatA and TatB from *E. coli*. The arrows at the top highlight residues that were selected for replacement with alanine (aspartic acid in case of P2, or glycine in case of A31). **(C)** Schematic representation of the generally proposed N_{out}-C_{in} membrane topology of TatAy. The positions of replaced residues are indicated with arrows. It should be noted however that some studies provide support for the existence of an N_{in}-C_{out} topology of TatAy (not shown) that might, for example, exist transiently during the protein translocation reaction [49,50]. **(D)** Helical wheel projection of the amphiphatic helix of TatAy. Substituted residues are indicated with arrows.

Remarkably, all mutant TatAy proteins facilitate at least low levels of YwbN secretion in cells grown in medium without added NaCl. This shows that the respective mutant TatAyCy translocases are conditionally salt-sensitive. Remarkably, subsequent experiments reveal that the TatAC translocases from *Bacillus cereus* and *Staphylococcus aureus* are also salt-sensitive when expressed in *B. subtilis*. Taken together, our findings indicate that TatAC translocases are intrinsically salt-sensitive.

RESULTS

Identification of functionally important TatAy residues.

To assess the roles of conserved residues in TatAy, we mutated those residues that had previously been shown to be important for TatA function in *E. coli* (Fig. 1; [42-46,48,51]. Residues were substituted with Ala, except for Pro2 which was also replaced with Asp, and Ala31 which was replaced with Gly. The resulting mutant proteins were constitutively expressed from plasmid pHB201, which results in the expression of TatAy at levels that are comparable to the regular TatAy expression levels in *B. subtilis* strain 168 grown in LB medium with 1% NaCl (data not shown).

Since amino acid substitutions can have detrimental effects on protein stability, we investigated the production of TatAy mutant proteins by Western blotting using cells from overnight cultures in LB medium with 1% NaCl. As shown in Figure 2A, only the P2D, K22A and P25A mutants could not be detected, whereas all other mutant TatAy proteins were produced, albeit to slightly varying levels. Strains producing the K22A or P25A mutant TatAy proteins showed growth defects, suggesting that the expression of these mutant proteins is detrimental to the cells. Detailed further analyses revealed that both the K22A and P25A proteins were detectable in strains freshly transformed with plasmids encoding the respective mutant TatAy proteins, but that the production of K22A and P25A TatAy was rapidly lost upon cultivation of these transformants (data not shown). While the K22A TatAy protein appeared to be produced at very low levels, the P25A TatAy protein was produced at levels that are comparable to the wild-type TatAy (Table I). Interestingly, the G20A mutation resulted in a significantly elevated cellular TatAy level, while the A31G mutation resulted in a significantly decreased level of TatAy (Fig. 2A). These findings suggest that the P2D, K22A and A31A are more prone to degradation than other mutant TatAy proteins or wild-type TatAy, and that the G20A TatAy mutant is more stable than the wild-type TatAy. To test the functionality of the mutant TatAy proteins, the secretion of YwbN-Myc was monitored by Western blotting. Several amino acid substitutions resulted in a drastically reduced secretion of YwbN (Fig 2B). Specifically, no YwbN secretion was detectable for strains producing the P2A, P2D, G20A, P21A, L24A or G28A mutant TatAy proteins. Also, no YwbN secretion was detectable for strains producing the consistent with the absence of TatAy production (Fig. 2A). We therefore verified in additional experiments that both mutant proteins were unable to sustain effective YwbN secretion (Table I; data not shown). Compared to strains producing the wild-type TatAyCy protein, reduced levels of

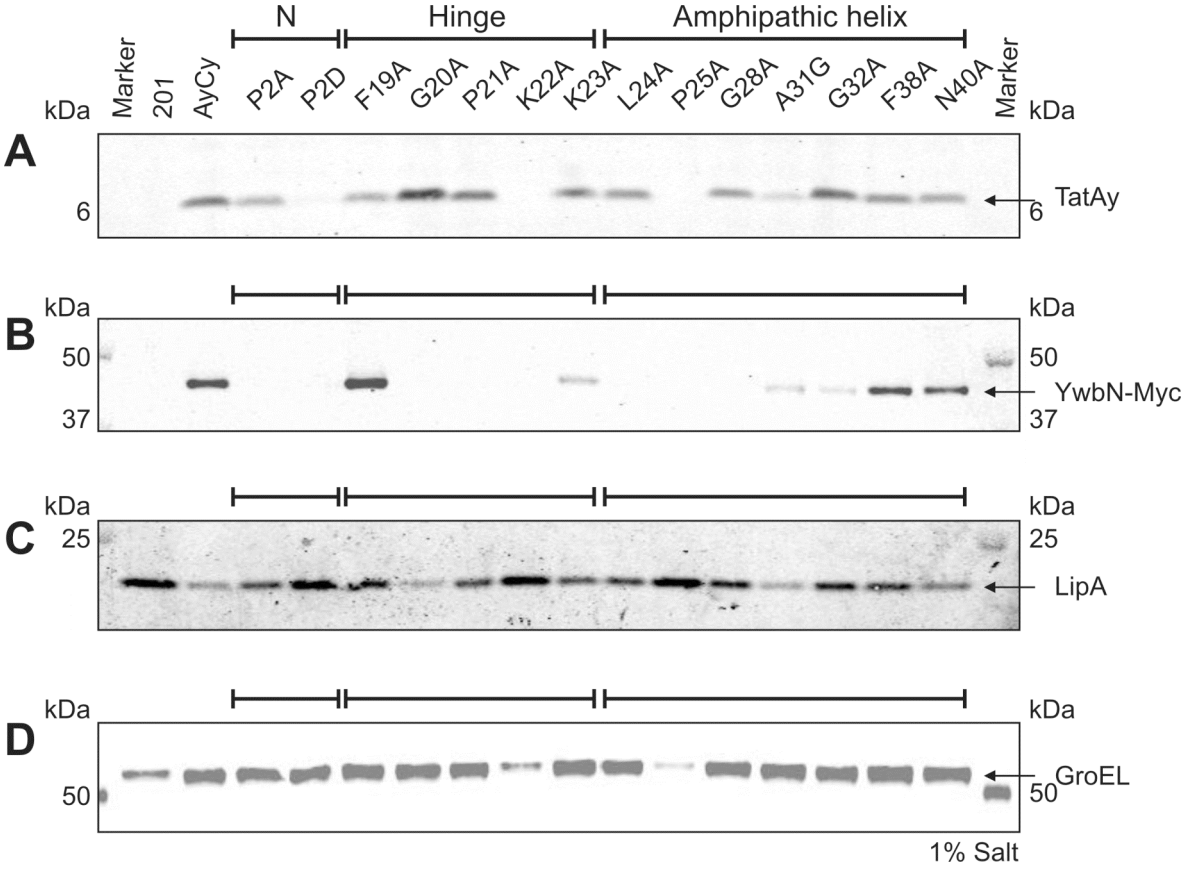


Figure 2. Effects of amino acid replacements on the stability and translocation activity of TatAy in *B. subtilis*. (A) TatAy mutant proteins detected by Western blotting in *B. subtilis tatAyCy* mutant cells from overnight cultures in LB medium with 1% NaCL. The cells contained pHB201 carrying a mutant *tatAy* gene and a wild-type *tatCy* gene. Cells containing the empty pHB201 vector, or pHB201 carrying the wild-type *tatAyCy* genes were used as controls. **(B)** Export of YwbN-Myc after 3 hours of xylose-induced expression of *ywbN-myc* by cells grown in LB medium with 1% NaCL. **(C)** LipA secretion as determined 6 hours after dilution from an overnight culture in LB medium with 1% NaCl. **(D)** GroEL release into the growth medium by cells grown as described for panel B.

YwbN secretion were observed for strains producing the K23A, A31G, G32A, F38A or N40A proteins. Unexpectedly, the F19A mutation in TatAy resulted in an increase of YwbN secretion by about 30% (Fig. 2B). The esterase LipA is secreted mainly via the Sec pathway when produced at physiological levels [27]. However, when produced at very high levels the LipA protein is secreted largely Tat-dependently via an overflow mechanism [27,41]. Under these conditions, the TatAyCy translocase interferes to some extent with the secretion of LipA. Therefore, we also monitored LipA secretion by strains producing mutant TatAy proteins. Consistent with our previous findings, Western blotting analyses showed that production of wild-type TatAyCy resulted in slightly reduced levels of LipA secretion (Fig. 2C). Interestingly, the expression of different mutant TatAyCy translocases had different

effects on LipA secretion. The production of the N40A TatAy mutant resulted in lowered LipA secretion levels that was comparable to that observed when wild-type TatAy was produced. LipA secretion was also reduced in mutants G20A and A31G. The production of all other TatAy mutant proteins resulted, however, in increased LipA secretion levels (Fig. 2C; Table I). The LipA secretion levels of strains carrying plasmids that specify the P2D, K22A or P25A mutant proteins were comparable with those observed for the strain carrying the empty vector pHB201, which is consistent with the fact that these mutant proteins were not detectably expressed in the experiment shown in Figure 2. Remarkably expression of the F19A TatAy mutant resulted in improved secretion of both LipA and YwbN (Fig. 2, A and B). It should be noted that equal amounts of extracellular protein were loaded in the gels used for the Western blotting experiments in Figure 2. Consistent with this, most fractions contained equal amounts of the cytoplasmic lysis marker protein GroEL (Fig. 2D), except for the strain carrying the empty plasmid and the strains carrying plasmids specifying the TatAy mutant proteins K22A or P25A that were not detectably produced in this particular experiment (Fig. 2A). This indicates that the plasmid-directed expression of wild-type or mutant TatAyCy translocases induced a certain (low) degree of cell lysis. Together, these findings show that several mutant TatAy proteins that do not facilitate YwbN secretion can still exert a negative influence on the secretion of LipA. This suggests that these mutant

Table I. Effects of single amino acid substitutions on TatAy properties

B. subtilis TatAy	TatAy Stability	YwbN Secretion	LipA Secretion	Inactive or Unstable
ΔTat			+++	
WT	+++	+++	+	Active
P2A	++	-	++	Inactive
P2D	-	-	+++	Unstable
F19A	++	+++++	+++	Active
G20A	+++++	-	+	Inactive
P21A	+++++	-	++	Inactive
K22A	+	+	+++	Active
K23A	+++	+	++	Active
L24A	+++	-	++	Inactive
P25A	+++	-/+	+++	Active
G28A	+++	-	++	Inactive
A31G	+	+	+	Active
G32A	+++	+	++	Active
F38A	+++	++	++	Active
N40A	+++	++	+	Active

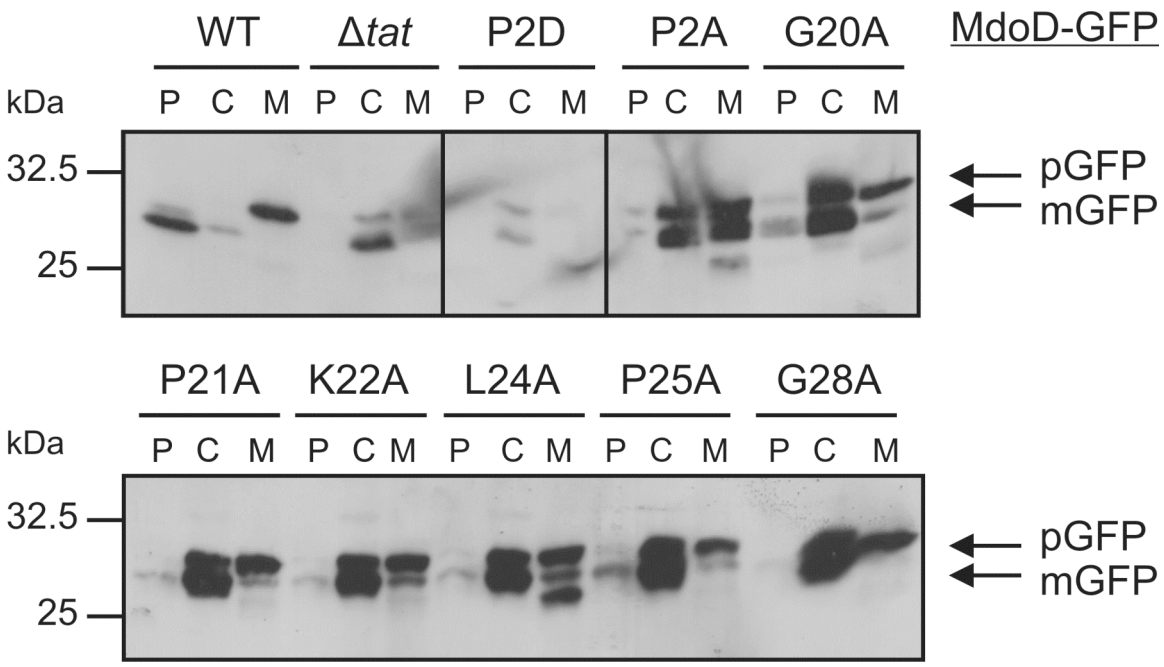


Figure 3. Effects of amino acid replacements on the translocation activity of TatAy in *E. coli*. The translocation activity of TatAyCy translocases with mutant TatAy subunits was assessed in *E. coli* using the hybrid MdoD-GFP precursor protein. Cells producing the wild-type TatAyCy translocase or no Tat translocase were used as controls. The presence of MdoD-GFP was monitored in periplasmic (P), cytoplasmic (C), or membrane (M) fractions. Precursor (pGFP) and mature (mGFP) forms of MdoD-GFP are indicated.

TatAy proteins are capable of TatAyCy complex formation and competitive binding of the LipA precursor, which might then result in lowered LipA secretion via Sec.

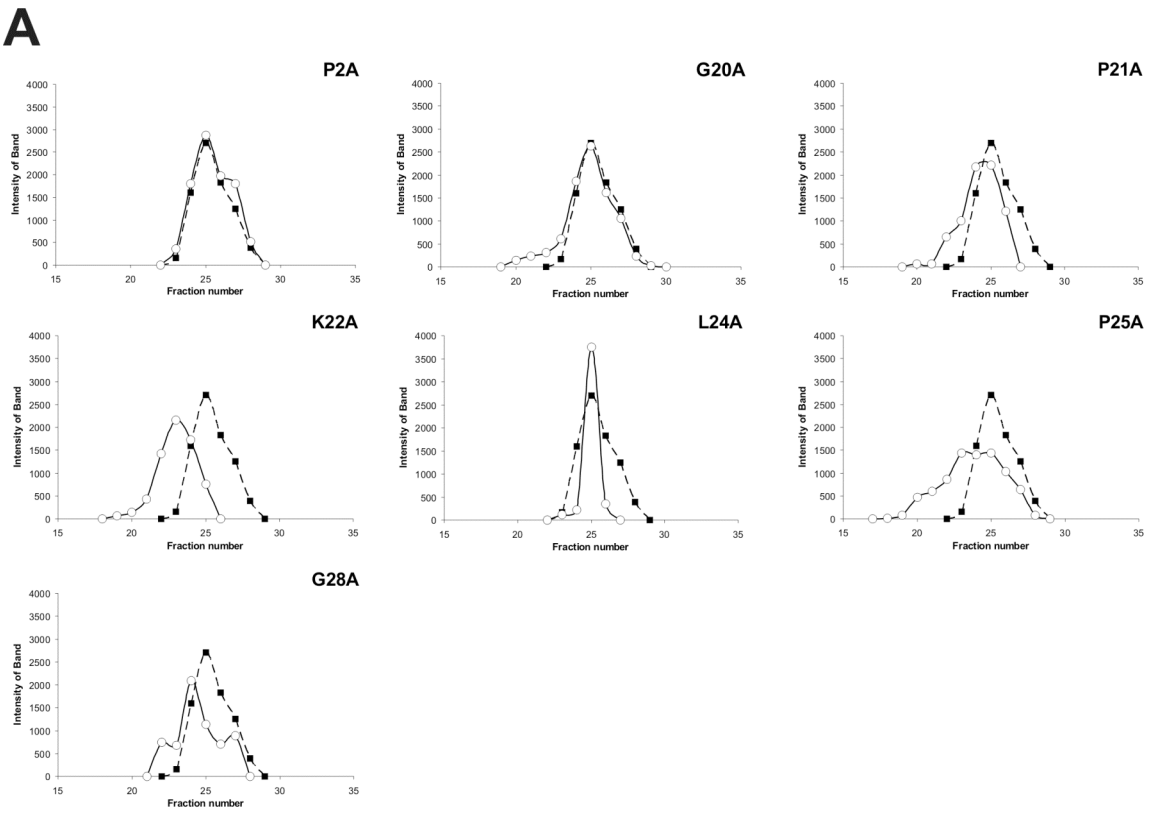
Activity of mutant TatAy proteins in *E. coli*.

To further assess the (in-)activity of the P2D, P2A, G20A, P21A, K22A, L24A, P25A and G28A TatAy mutant proteins, the respective mutant *tatAy-tatCy* operons were expressed in *E. coli* and their activity was monitored using the green fluorescent protein (GFP) fused to the RR-signal peptide of the *E. coli* MdoD protein. This hybrid MdoD-GFP precursor was previously shown to be effectively translocated in an *E. coli tatABCDE* deletion mutant upon co-expression of the *B. subtilis* TatAyCy translocase [52]. To study MdoD-GFP export to the periplasm of *E. coli* by mutant TatAyCy translocases, the *mdoD-gfp* gene was expressed from pBAD24, and the different mutant *tatAy-tatCy* operons were co-expressed from the compatible pEXT22 vector. Next, periplasmic, membrane and cytoplasmic cell fractions were analyzed by Western blotting using GFP-specific antibodies. No mature GFP was detected in the periplasmic fractions of strains producing the P2D or G28A TatAy mutant proteins (Fig. 3).

Strains producing the P2A, G20A, P21A, K22A, L24A or P25A TatAy mutants contained very low amounts of GFP in the periplasm, reflecting a severely impaired translocation activity (Fig. 3). Interestingly, strains producing the P2A, G20A, P21A, K22A, L24A, P25A, or G28A TatAy mutants contained relatively high amounts of precursor and mature-sized GFP in the membrane, while this was not the case when no Tat proteins were produced (Fig. 3). Together, these findings suggests that the respective mutant TatAyCy translocases were able to interact with the MdoD-GFP precursor and to promote its binding to the membrane. However, these mutant translocases were unable to facilitate effective transport to the periplasm. Strains producing the P2D TatAy protein did not contain membrane-associated MdoD-GFP, suggesting that the respective TatAyCy translocase was completely inactive or that insufficient amounts of P2D TatAy were produced. Furthermore, the strain producing the L24A TatAy mutant protein contained an abundant GFP degradation product in the membrane fraction. Similarly sized GFP degradation products were also detectable in the membrane fractions of strains producing other mutant TatAy proteins (e.g. P2A), albeit at much lower amounts (Fig. 3). Such degradation products were not detectable when the wild-type TatAyCy translocase was produced, suggesting that MdoD-GFP was prone to specific degradation when the L24A or P2A TatAy mutant proteins were produced.

Complex formation by mutant TatAy proteins.

To investigate whether mutant TatAy proteins, which do not facilitate YwbN secretion, do engage in TatAyCy and TatAy complexes as was previously shown for wild-type TatAy [52], the respective mutant *tatAy-tatCy* operons were expressed in a *tatABCDE* mutant strain of *E. coli* using the plasmid pBAD24. It should be noted that complex formation had to be studied in *E. coli*, because TatAy and TatCy could not be produced in sufficiently high amounts in *B. subtilis* to allow detection of complexes by affinity purification or blue-native PAGE (data not shown). To characterize the TatAyCy complexes expressed in *E. coli* by affinity chromatography and subsequent gel filtration as previously described (Barnett *et al*. 2009), a Strep-II tag was fused to the C-terminus of TatCy. Next, membranes of strains producing P2A, G20A, P21A, K22A, L24A, P25A or G28A mutant TatAy proteins and TatCy-strep were isolated, solubilized in digitonin, and loaded on a Streptactin column for affinity purification. After elution from the column, the proteins were concentrated and loaded on a Superose-6HR gel filtration column to assess the sizes of formed TatAyCy complexes. The collected elution fractions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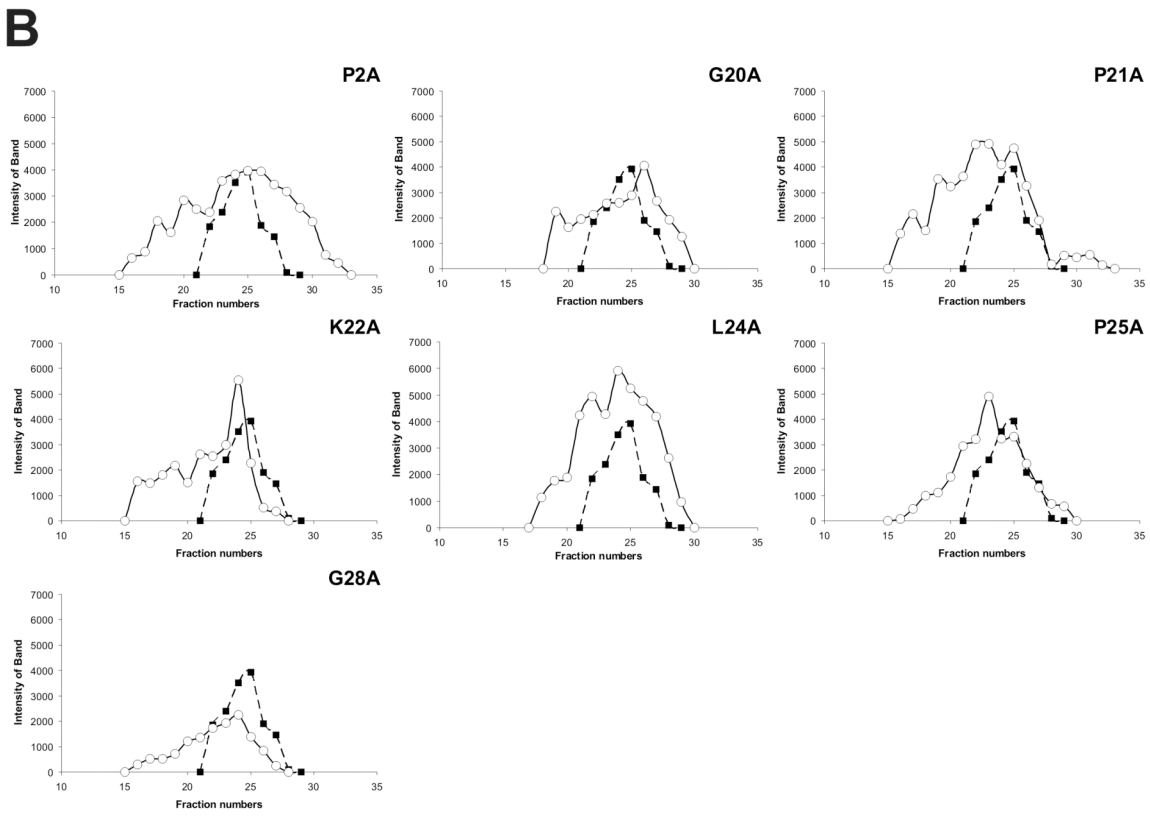
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Figure 4. Complex formation by mutant TatAy proteins. (A) Mutant *tatAy-tatCy-strep*II operons were expressed in a *tatABCDE* mutant strain of *E. coli*. Isolated membranes were solubilized in digitonin, and loaded on a Streptactin column for affinity purification. After elution from the column, the proteins were concentrated and loaded on a Superose-6HR gel filtration column. The collected elution fractions were analyzed by Western blotting with specific antibodies against TatAy or the Strep-II tag attached to TatCy. Immunoblots of all elution fractions of membrane-localized TatAy that was co-purified with TatCy-Strep were analyzed by densitometry. Intensities of the bands were plotted against fraction number. The column was calibrated using a set of protein standards of known molecular weight, namely thyroglobin (669 kDa), ferritin (440 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7kDa). Each plot shows the elution patterns for wild-type TatAyCy-Strep (filled squares) and a TatAyCy-Strep complex containing a particular TatAy mutant protein (empty circles). **(B)** To analyze TatA complexes formed by mutant TatAy proteins, the flow-through fractions obtained after Streptactin affinity chromatography of solubilized membranes containing mutant TatAyCy complexes (see panel A) were applied to a Superose-6HR gel filtration column. The collected gel filtration fractions were subsequently analyzed by Western blotting with antibodies against TatAy. Immunoblots of all elution fractions of membrane-localized TatAy were analyzed by densitometry and band intensities were plotted against fraction number. Each graph shows the wild-type TatAy complexes (filled squares) and complexes obtained for mutant TatAy protein (empty circles).

were analyzed by Western blotting with antibodies against TatAy or the Strep-II tag that was attached to TatCy. Overall, only little differences could be observed in the elution patterns of TatAyCy complexes containing wild-type TatAy or the P2A, G20A, P21A, L24A or G28A mutant TatAy proteins (Fig. 4A). In contrast, the TatAyCy complexes containing the K22A or P25A mutant TatAy proteins seemed to be substantially larger than the wild-type TatAyCy complexes. To study the properties of mutant TatAy complexes, the flow-through fractions obtained after Streptactin affinity chromatography of solubilized membranes from strains producing the P2A, G20A, P21A, K22A, L24A, P25A orG28A mutant TatAy proteins were separated on a Superose-6HR gel filtration column. The collected elution fractions were subsequently analyzed by Western blotting with antibodies against TatAy. Interestingly, compared to the wild-type TatAy complexes, quite substantial variations were observed for the elution patterns of the mutant TatAy complexes (Fig. 4B). All mutant TatAy proteins tested formed larger complexes than wild-type TatAy, and this effect was most pronounced for the complexes formed by the P2A, P21 and K22A mutants. Moreover, the P2A, P21A and L24A TatAy mutants formed also complexes that were substantially smaller than the complexes formed by wild-type TatAy. Taken together, the results of our translocation assays in *B. subtilis* and *E. coli*, and the analyses on complex formation, indicate that the P2A, G20A, P21A, K22A, L24A, P25A and G28A TatAy mutants are defective in

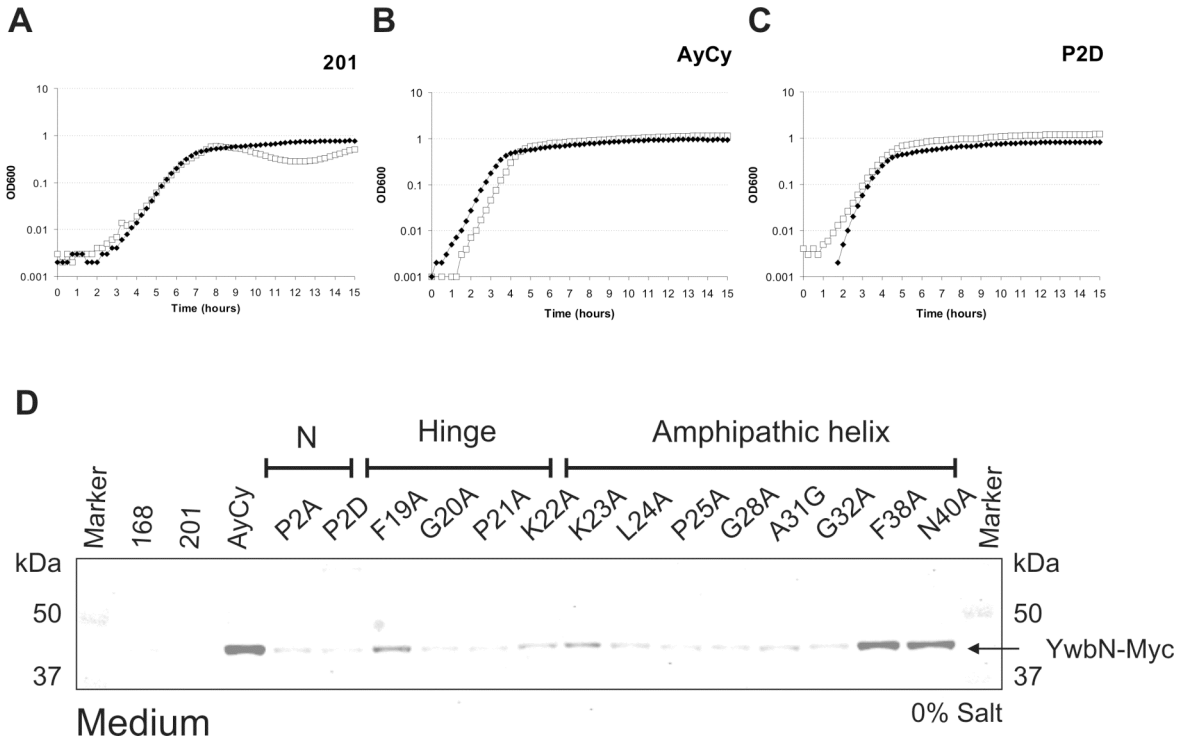

Figure 5. Activity of TatAy mutant proteins produced by *B. subtilis* cells grown in LB medium without salt. *B. subtilis tatAyCy* mutant cells transformed with the empty pHB201 vector (**A**, open squares), pHB201 carrying the wild-type *tatAyCy* genes (**B**, open squares), or pHB201 carrying the mutant P2D *tatAy* gene plus a wild-type *tatCy* gene (**C**, open squares) were grown in LB medium without added salt. Growth was monitored for 15 h by optical density readings at 600 nm (OD_{600}). Growth of the parental strain 168 is indicated by filled diamonds. **(D)** YwbN-Myc secretion by *tatAyCy* mutant strains producing the indicated plasmid-encoded mutant TatAy plus wild-type TaCy was monitored by Western blotting as in Figure 2.

translocation activity rather than complex formation.

Mutant TatAyCy translocases are conditionally salt-sensitive in *B. subtilis*.

B. subtilis strains lacking functional *tatAyCy* genes show a strong growth defect when cultivated in LB medium without salt (Fig. 5A). This is due to a requirement of YwbN secretion in the acquisition of iron under these low salinity growth conditions (chapter 4). The fact that the low salinity growth phenotype of *tatAyCy* mutants can be rescued by introducing plasmid-borne copies of the *tatAyCy* genes provides a facile assay for the activity of mutant TatAy proteins (Fig. 5B). Intriguingly, all TatAy mutants generated in this study were able to fully complement the growth defect of a *tatAyCy* deficient strain (Fig. 5C; only the result for complementation with the P2D TatAy mutant is shown). This suggested that the mutant TatAy proteins would engage in translocation competent TatAy and TatAyCy complexes when the producing cells were grown in LB without

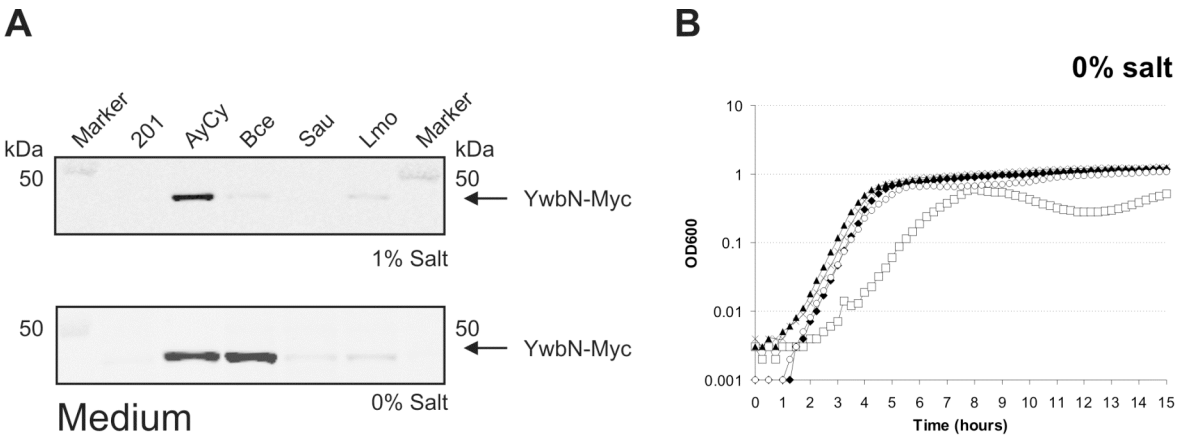

Figure 6. Salt-sensitivity of the *Bacillus cereus* and *Staphylococcus aureus* TatAC translocases produced in *B. subtilis*. (A) *B. subtilis tatAyCy* mutant cells transformed with the empty pHB201 vector (201), pHB201 carrying the wild-type *tatAyCy* genes (AyCy), pHB201 carrying the *B. cereus tatAC* genes (Bce), pHB201 carrying the *S. aureus tatAC* genes (Sau), or pHB201 carrying the *L. monocytogenes tatAC* genes (Lmo) were grown in LB medium with 1% NaCl (upper panel) or LB medium without added NaCl (lower panel). Secretion of YwbN-Myc was monitored by Western blotting as in Figure 2. **(B)** Growth of the *tatAyCy* mutant *B. subtilis* strains producing the TatAC translocases of *B. cereus* (filled triangles), *S. aureus* (empty circles), or *L. monocytogenes* (crosses) was monitored for 15 h by optical density readings at 600 nm (OD_{600}). *B. subtilis tatAyCy* mutants transformed with the empty pHB201 vector (empty squares), or the wild-type *tatAyCy* genes (filled diamonds) were used as controls.

salt. This idea was tested by studying the secretion of YwbN-Myc in *tatAyCy* mutant cells with plasmid-borne copies of a mutant *tatAy* gene plus a wild-type *tatCy* gene. Indeed, all strains producing the P2A, P2D, G20A, P21A, K22A, L24A, P25A or G28A mutant TatAy proteins secreted low but detectable amounts of YwbN when grown in LB medium without salt (Fig. 5D). This confirms the conclusion from the growth complementation experiments that these mutant TatAy proteins can form active TatAyCy translocases. Furthermore, these findings reveal that the TatAyCy translocases formed by the mutant TatAy proteins are conditionally salt-sensitive.

TatAC translocases from *B. cereus* and *S. aureus* are salt-sensitive when produced in *B. subtilis*

The observation that mutant TatAyCy translocases are salt-sensitive opened up the possibility that this is a more general feature of TatAC-type translocases from Gram-positive bacteria. We therefore investigated whether this might be the case for TatAC translocases from *B. cereus*, *S. aureus* and *L. monocytogenes* when heterologously expressed in *B. subtilis*. To this end, the corresponding genes were amplified by PCR and cloned in the pHB201 plasmid for expression in a *B. subtilis tatAyCy* mutant strain. As shown in

Figure 6A, The TatAC translocases of *B. cereus* and *L. monocytogenes* facilitated low-level secretion of YwbN when the *B. subtilis tatAyCy* mutant cells expressing these heterologous translocases were grown in LB medium with 1% salt, while the TatAC translocase of *S. aureus* did not display this complementing activity under these conditions. Remarkably, YwbN secretion by the *B. cereus* TatAC translocase was drastically increased when *B. subtilis tatAyCy* mutant cells expressing this translocase were grown in LB medium without salt. Also, secretion of YwbN by the TatAC translocase of *S. aureus* could be demonstrated for *B. subtilis tatAyCy* mutant cells grown in medium without salt, albeit at a much lower level than was detected for the *B. cereus* TatAC translocase. Accordingly, both the TatAC translocases of *B. cereus* and *S. aureus* complemented for the growth defect of the *B. subtilis tatAyCy* strain in LB medium without salt (Fig. 6B). Notably, the activities of the *L. monocytogenes* TatAC translocase and the wild-type TatAyCy translocase of *B. subtilis* were not affected by the presence or absence of 1% salt in the growth medium (Fig. 6, A and B). This in fact shows that the salinity of the growth medium has no impact on the competence of the YwbN protein for Tat-dependent translocation, but rather on the activities of particular TatAC translocases that were expressed in *B. subtilis*. Taken together our findings show that TatAC translocases of *B. cereus* and *S. aureus* are intrinsically salt-sensitive.

DISCUSSION

Studies on the structure-function relationships in bifunctional TatA subunits of TatAC translocases from Gram-positive bacteria have so far been lacking. In the present studies, we have identified residues of the bifunctional TatAy subunit of *B. subtilis* that are important for activity of the corresponding TatAyCy translocase. Most mutations in TatAy caused severely decreased translocation activity, as was shown by monitoring the secretion of the TatAyCy substrate YwbN into the growth medium of *B. subtilis* or the export of the TatAyCy substrate MdoD-GFP to the periplasm of *E. coli*. A remarkable exception was formed by an F19A substitution in the hinge region of TatAy that triggered an enhanced YwbN secretion via TatAyCy. Gel filtration experiments revealed no clear alterations in TatAyCy complex formation by mutant TatAy proteins, but an increased heterogeneity was observed in the mutant TatAy protomers. Interestingly, all mutations in

TatAy rendered the TatAyCy translocase conditionally NaCl-sensitive suggesting that this might be an intrinsic property of TatAC translocases. Indeed, studies on heterologously expressed TatAC translocases from *B. cereus* and *S. aureus* revealed that these particular translocases are intrinsically salt-sensitive, at least when produced in *B. subtilis*. In contrast, the TatAC translocase of *L. monocytogenes* did not reveal the salt-sensitive phenotype, like the wild-type TatAyCy translocase of *B. subtilis*.

Previous studies in *E. coli* have shown that TatA is a remarkably flexible protein that can tolerate many changes in its structure [42,44,45,48]. It was therefore no major surprise that the TatAy protein turned out to be remarkably stable upon site-specific mutagenesis. Notably, the only mutation in TatAy resulting in a highly unstable TatAy protein was the N-terminal P2D substitution. This finding suggests that Pro2 may have a role in stabilizing the transmembrane helix of TatAy at the extracytoplasmic side of the membrane (Fig. 1), and that the negatively charged side chain of Asp somehow interferes with helix stability. A P2A substitution did not cause TatAy instability, showing that the small and hydrophobic side chain of Ala can be tolerated at this position. Notably, a G3D substitution in *E. coli* TatA rendered this protein bifunctional [53], whereas the equivalent P2A substitution in TatAy rendered the TatAyCy translocase inactive.

The five highly conserved residues of the TatAy hinge region turned out to be of major importance for TatAy function as their substitution with Ala rendered the TatAyCy translocase either more active (F19A), severely impaired in activity (K23A), or completely inactive (G20A, P21A, K22A). Most likely, these substitutions change the flexibility of the hinge region such that translocase activity is impaired. Especially, the G20A substitution will make the hinge less flexible as Gly residues generally contribute to structural flexibility of polypeptide chains. Conversely, the P21A substitution would make the hinge more flexible. Likewise, replacement of the positively charged Lys22 or Lys23 residues with Ala may cause an increased flexibility of the hinge as these two Lys residues may engage in interactions with negatively charged phospholipids in the membrane (Fig. 1). These findings are largely consistent with studies on *E. coli* TatA were a substitution of Phe20 (equivalent to Phe19 in TatAy) rendered the *E. coli* TatABC translocase largely inactive (Table II). Unfortunately, the roles of conserved residues in TatB of *E. coli* have been studied less extensively so comparisons of the effects of mutations in TatB and TatAy are only possible for a few residues. Clearly, there are differences as substitutions in Gly21 of TatB (equivalent to Gly20 of TatAy) affect TatB

Table II. Effects of single amino acid substitutions in *B. subtilis* TatAy or *E. coli* TatA on translocation activity. References used for Table II are [42,44,45,48,53]. * = in absences of TatB [53].

***B. subtilis* TatAy**	**YwbN Secretion**	**TorA Secretion**	**TorA Secretion**	***E. coli* TatA**
Substitution	Ala/Gly	Ala	Cys	Substitution
Tat system	TatAyCy	TatABC	TatABC	Tat system
WT	+++	+++	+++	
P2A	-		++	G3C
P2D	-	+*		G3D
F19A	+++++	++ / -	++	F20
G20A	-	+++ / -	-	G21
P21A	-		+	T22
K22A	+		-	K23
K23A	+	+++	-	K24
L24A	-	+	-	L25
P25A	-/+		++	G26
G28A	-		-	G29
A31G	+		-	L32
G32A	+	+	-	G33
F38A	++	-	-	F39
N40A	++	++	++	K41

Table III. Effects of single amino acid substitutions in *B. subtilis* TatAy or *E. coli* TatB on translocation activity. References used for Table III are [44,45,54].

***B. subtilis* TatAy**	**YwbN Secretion**	**TorA Secretion**	**TorA Secretion**	***E. coli* TatB**
Substitution	Alanine	Alanine	Cysteine	Substitution
Tat system	TatAyCy	TatABC	TatABC	Tat system
WT	+++	+++	+++	
P2A	-			D3
P2D	-		+++	D3
F19A	+++++		++	L20
G20A	-	+		G21
P21A	-			P22
K22A	+			Q23
K23A	+	++		R24
L24A	-	+++		L25
P25A	+/ -	+		P26
G28A	-			V29
A31G	+		+++	V32
G32A	+		++	A33
F38A	++		+	L39
N40A	++		++++	S41

activity (Table III), the corresponding mutation G20A in TatAy completely inactivated TatAyCy function. Similar differences are observed for substitutions Arg24 and Leu25 in TatB.

Our site-directed mutagenesis studies seem to divide the cytoplasmic amphipathic helix of TatAy into an essential N-terminal part and less important C-terminal part, although it should be noted that not all residues of the amphipathic helix were substituted in the present studies. Clearly, the tested substitutions of the N-terminal helix residues Leu24, Pro25, Gly28 and residues Ala31 and Gly32 had drastic effects on translocation activity, while this was not the case for the C-terminal helix residues Phe38 and Asn40. These findings are consistent with the results of mutagenesis studies on TatA of *E. coli*, except for substitutions in Phe39 (equivalent to Phe38 of TatAy), which rendered the TatABC translocase of *E. coli* inactive (Table II). By contrast, mutations in *E. coli* TatB that are equivalent to the presently tested mutations in TatAy had very little effects on TatB function (Table III). The only exception is an L39C substitution in TatB, which resulted in reduced translocation activity of the *E. coli* TatABC translocase, unlike the equivalent F38A mutation in TatAy, which had only a minor effect on TatAyCy function (Table III). These findings clearly underscore the view that *E. coli* TatA and *B. subtilis* TatAy have functionally equivalent roles in RR-precursor translocation. In this respect it is noteworthy that the tested TatAy mutants appear not to cause defects in precursor binding by TatAyCy, as can be deduced from the studies on LipA secretion in *B. subtilis* and MdoD-GFP translocation in *E. coli*. This is consistent with the view that a TatAy-TatCy subcomplex serves in precursor reception [30], like the TatB-TatC subcomplexes of the *E. coli* and thylakoidalTatABC translocases [32-34]. Indeed, TatAyCy complex formation was not significantly affected by most TatAy mutations tested and, in those cases where effects were observed on the mass distribution of TatAyCy complexes (K22A and P25A), there were still substantial amounts of complexes with wild-type mass properties detectable (Fig. 4A).

Interestingly, most of the tested mutations in TatAy resulted in the formation of heterogeneously sized TatAy complexes. This suggests that the respective mutations, in particular the P2A, P21A, G28A and L24A substitutions, cause defects in TatAy complex assembly and/or the specific interactions needed for transfer of precursors from the presumed TatAy-TatCy RR-receptor complex to the translocation channel that is probably formed by TatAy protomers. If this view is correct, it means that the interactions between the mutant TatAyCy-precursor complexes and the mutant TatAy complexes in the membrane

are salt-sensitive, since all mutant TatAy proteins did engage in translocation competent TatAyCy complexes when cells were grown in medium of low salinity. As confirmed by the observed salt-sensitivity of the TatAC translocases from *B. cereus* and *S. aureus* upon expression in *B. subtilis*, salt-sensitivity may be an intrinsic of important electrostatic interactions during the formation of active TatAC channels for the transfer of proteins from the TatAC receptor complex into the TatA channel. It is presently not clear why some wild-type translocases like TatAC of *B. cereus* and *S. aureus* are salt-sensitive, whereas other wild-type translocases like TatAyCy of *B. subtilis* and TatAC of *L. monocytogenes* do not share this property. However, this may relate to the high salt tolerance that has evolved in the latter organisms (chapter 4; [55]). In any case, the finding that cells expressing wild-type TatAC translocases of *B. subtilis* or *L. monocytogenes* showed no altered YwbN secretion in response to changes in the salt content of the growth medium indicates that the salt-sensitive YwbN secretion by the TatAC translocases of *B. cereus* or *S. aureus* relates to properties of the respective translocases rather than salt-induced changes in the YwbN substrate protein.

In conclusion, the present finding that mutant and wild-type TatAC translocases are intrinsically salt-sensitive provides novel leads for further mechanistic, structural and applied studies on these intriguing systems for protein translocation. For future studies it will definitely be interesting to investigate whether mutations in TatC, as were recently published by Eijlander *et al*. (2009) [56], will render TatAC-type translocases salt-sensitive as well, or whether this is an exclusive feature of certain TatA mutants. The identification of mutations (e.g. G20A) that stabilize TatAy may be of particular use in structural analyses, while mutations that enhance TatAC activity (e.g. F19A) may facilitate future biotechnological applications of engineered Tat pathways.

MATERIALS AND METHODS

Plasmids, bacterial strains and media.

Plasmids are listed in Table IV and strains in Table V. The standard LB medium that was also used in all our previous studies contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). For some experiments NaCl was omitted from the LB medium (0% NaCl). When required, media for *E. coli* were supplemented with ampicillin (Amp; 100 μg/ml), erythromycin (Em; 100 μg/ml), kanamycin (Km; 20 μg/ml), chloramphenicol (Cm; 5 μg/ml) or spectinomycin (Sp; 100 μg/ml); media for *B. subtilis* were supplemented with Em (2 μg/ml), Km (20 μg/ml), Cm (5 μg/ml) or Sp (100 μg/ml).

DNA techniques.

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described [57]. Enzymes were from Roche Molecular Biochemicals or New England Biolabs. *B. subtilis* was transformed as described [58]. PCR was carried out with Pwo DNA polymerase. All constructs were sequenced to confirm correctness.

Construction of tatAy mutations and tatAyCy complementation plasmids.

Particular codons in *tatAy* were replaced by site-directed mutagenesis, using PCR primers with one or more nucleotide changes. Table VI lists all the primers used in this study. In a first PCR, the 5' part of *tatAy* was amplified using the forward primer JW01Ay2 and a reverse primer specifying one or two nucleotide changes in *tatAy*. The resulting PCR product was purified using the Roche PCR purification kit. This PCR product was then used as a forward primer with JW04Cy2 as the reverse primer to create a second PCR product, specifying the complete *tatAyCy* operon with a mutant *tatAy* gene. Mutant *taAyCy* operons were cloned in pUC18 and the correctness of the amplified sequences was verified. For expression and activity studies in *B. subtilis*, the mutant *tatAyCy* operons were excised from pUC18 using *Hin*dII and *Eco*RI, and ligated into the *Sma*I and *EcoR*I restricton sites of the *E. coli-B. subtilis* shuttle vector pHB201. To study TatAyCy complex formation in *E. coli*, mutant *tatAyCy* operons were transferred to pBAD24. For this purpose, the mutant *tatAyCy* operons were amplified by PCR with a 3' primer specifying a *strep*-II tag that is coupled to the 3' end of *tatCy*. The resulting PCR products were cleaved with *Bsm*BI and *Xba*I, and ligated to pBAD24 that had been cleaved with *Bsm*BI and *Nco*I. To study TatAyCy-facilitated export of MdoD-GFP in *E. coli*, the low-expression vector pEXT22 was used. To this end, the mutant *tatAyCy* operons were amplified by PCR, cleaved with *Hin*dIII

and *Eco*RI, and ligated to *Hin*dIII-*Eco*RI- cleaved pEXT22.

Table IV. Plasmids

Plasmids	Relevant properties	Reference
B. subtilis		
pHB201	Contains multiple cloning site to place genes under the control of the p59 promoter; 6.6 kb; Em^r; Cm^r	This study
pHB-SDMP2A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single P2A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMP2D	pHB201-derivative containing the *tatAy-tatCy* operon; with a single P2D substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMF19A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single F19A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMG20A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single G20A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMP21A	pHB201-derivative containing the *tatAy-tatCy* operon; with a double F19A and P21A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMK22A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single K22A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMK23A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single K23A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDML24A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single L24A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMP25A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single P25A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMG28A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single G28A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMA31G	pHB201-derivative containing the *tatAy-tatCy* operon; with a single A31G substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMG32A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single G32A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMF38A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single F38A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-SDMN40A	pHB201-derivative containing the *tatAy-tatCy* operon; with a single N40A substitution in TatAy; 7.6 kb; Em^r; Km^r	This study
pHB-AyCy	pHB201-derivative containing the *tatAy-tatCy* operon; 7.6 kb; Em^r; Km^r	This study
pHB-Bce	pHB201-derivative containing the *B. cereus tatA-tatC* operon; 7.6 kb; Em^r; Km^r	This study
pHB-Sau	pHB201-derivative containing the *S. aureus tatA-tatC* operon; 7.6 kb; Em^r; Km^r	This study
pHB-Lmo	pHB201-derivative containing the *L.monocytogenes tatA-tatC* operon; 7.6 kb; Em^r; Km^r	This study

Table IV. Plasmids continued

Plasmids	Relevant properties	Reference
<u>*E. coli*</u>		
pBAyCys	pBAD24-derivative containing the *B. subtilis tatAy-tatCy-strep*-II tag operon; 5.5 kb; Amp^r	Barnett *et al*., 2009
pBAD-P2As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single P2A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-P2Ds	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single P2D substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-G20As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single G20A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-P21As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single P21A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-K22As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single K22A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-L24As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single L24A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-P25As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single P25A substitution in TatAy; 5.5 kb; Amp^r	This study
pBAD-G28As	pBAD24-derivative containing the *tatAy-tatCy-strep*-II tag operon; with a single G28A substitution in TatAy; 5.5 kb; Amp^r	This study
pEXT-AyCy	pEXT22-derivative containing the *B. subtilis tatAy-tatCy* operon; 7.1 kb; Kan^r	Barnett *et al*., 2009
pEXT-P2A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single P2A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-P2D	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single P2D substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-G20A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single G20A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-P21A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single P21A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-K22A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single K22A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-L24A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single L24A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-P25A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single P25A substitution in TatAy; 7.1 kb; Kan^r	This study
pEXT-G28A	pEXT22-derivative containing the *tatAy-tatCy* operon; with a single G28A substitution in TatAy; 7.1 kb; Kan^r	This study
pBAD-MdoD-GFP	pBAD24-derivative containing *mdoD-gfp* gene hybrid; Amp^r	Barnett *et al*., 2009

Cloning of the *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* tatAC genes.

Primers for amplification of the *tatAC* genes from *Bacillus cereus, Listeria monocytogenes* EGD and *Staphylococcus aureus* RN4220 were designed from the respective genome sequences as deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome; Table VI). The amplified PCR products were cloned in pUC18 or pTOPO (Invitrogen) and sequenced. For expression in *B. subtilis*, correctly amplified *tatAC* genes from *B. cereus* and *S. aureus* were cloned in the *Eco*RI site of pHB201. The fragment with the *L. monocytogenes tatAC* operon was cleaved with *Bam*HI and *Xho*I, and ligated to pHB201 cleaved with the same enzymes.

Export assays for YwbN and MdoD-GFP.

B. subtilis strains lacking the *tatAyCy* genes were transformed with pHB201 plasmids carrying wild-type or mutant *tatAyCy* genes from *B. subtilis*, *B. cereus*, *S. aureus* or *L. monocytogenes*. To monitor activity of pHB201-encoded TatAC translocases, the XywbN-Myc cassette was integrated in the *amyE* locus, allowing xylose-inducible expression of Myc-tagged YwbN. Secretion of YwbN-Myc was assessed by transferring cells from an overnight culture to fresh medium. After 3 h of growth in fresh medium at 37°C, YwbN-Myc expression was induced by the addition of 1% xylose and 0,5 mM delta-aminolaevulinic acid to the cultures. Growth was continued for 3 h and, then, cells and growth medium fractions were separated

Table V. Strains

Strains	Relevant properties	Reference
E. coli		
DH5α	*supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook *et al.*, 1989
E. coli MC4100	F⁻ Δ*lacU*169 *araD*139 *rpsL*150 *rel*A1 *ptsF rbs flbB*5301	Casadaban *et al.*, 1976
E. coli MC4100 ΔtatABCDE	Clean knock-out of the *tatABCD* operon and the *tatE* gene	Wexler *et al.*, 2000
B. subtilis		
B. subtilis 168	*trpC2*	Kunst *et al.*, 1997
B. subtilis 168 tatAyCy X-ywbN	*trpC2*, *tatAy-tatCy*::Sp^r; *amyE*::*xylA-ywbN*-c-*myc*	Jongbloed *et al.*, 2004

Table VI. Primers

Primer	Sequence	Definition underline
TatAy substitutions		
pHB201		
TatAyP2AbsR	CAGGACCGATCGCCATAT	CCG > GCG
TatAyP2DbsR	GGACCGATGTCCATATTTGG	CCG > GAC
pCAyCysdm2F	CTGCCATTGAAATAGACC	
TatAyF19AbsR	TGGGACCGGCGATAATCA	TTC > GCC
AyG20AssR	CTTTTTGGGAGCGAAGATAAT	GGT > GCT
TatAyP21AR	GCTTTTTGGCACCGAAGA	CCC > GCC
TatAyK22AR	CAGCTTTGCGGGACCGAA	AAA > GCA
TatAyK23AR	TCAGGCAGCGCTTTGGGA	AAG > GCG
TatAyL24AR	CAATTCAGGCGCCTTTTTGG	CTG > GCG
TatAyP25AR	CCCCAATTCAGCCAGCTT	CCT > GCT
TatAyG28AR	GCTGCTTTCGCCAATTCA	GGG > GCG
TatAyA31GR	GTGTCTCCCCCTGCTTTC	GCG > GGG
TatAyG32AR	ACGAAGTGTGTCTGCCGC	GGA > GCA
TatAyF38AR	GCGTTTTTAGCTTCACGA	TTT > GCT
TatAyN40AR	AGCGGCTTTAAATTCACG	AAC > GCC
JW01Ay2	CCCCCGTCGACGAATAGAGGGAA	(*Sal*I)
JW04Cy2	CCCCCGAATTCTCGCATGAGAAATG	(*Eco*RI)
pBAD24		
RTEAyF	CGCGTCTCGCATGCCGATCGGTCCTGGAAGCCTTGCTG	(*Bsm*BI)
RTEAyF-SDMP2A	CGCGTCTCGCATGGCGATCGGTCCTGGAAGCCTTGCTG	CCG > GCG
RTEAyF-SDMP2D	CGCGTCTCGCATGGACATCGGTCCTGGAAGCCTTGCTG	CCG > GAC
JJystrep02	ATATTCTAGATTATTTTTCAAACTGTGGGTGCGACCAA TTCGATTGCCCAGAAGACACGTCCCG	(*Xba*I, StrepII tag)
pEXT22		
RTEAy-HindIII-F	CGAAGCTTGCATGCCGATCGGTCCTGGAAGCCTTGCTG	(*Hind*III)
RTEAyP2A-HindIII-F	CGAAGCTTGCATGGCGATCGGTCCTGGAAGCCTTGCTG	CCG > GCG
RTEAyP2D-HindIII-F	CGAAGCTTGCATGGACATCGGTCCTGGAAGCCTTGCTG	CCG > GAC
JW04Cy2	CCCCCGAATTCTCGCATGAGAAATG	(*Eco*RI)
TatAC systems		
pHB201		
LMEGDEXhoIF	GGGGGCTCGAGTTTGTACATGTTGTACCTCCC	(*Xho*I)
LMEGDEBamHIR	GGGGGGGATCCCACGAAGCGCTTAAGTTAACG	(*Bam*HI)
BC14579NdeIF	CCCCCCATATGAATGGCACCGATACCATGTG	(*Nde*I)
BC14579NcoIR	GGGGGCCATGGCTCCACCTAATTCACCTTCC	(*Nco*I)
SACOLNdeIF	GGGGGCATATGCAACGATTAACTGGTTGGGC	(*Nde*I)
SACOLNcoIR	GGGGGCCATGGATCGACTTGCAAGACCAAGC	(*Nco*I)

by concentrated 20-fold upon precipitation with trichloroacetic acid (TCA). The presence of YwbN-Myc in the different fractions was monitored by Western blotting and immunodetection.

The activity of mutant TatAyCy translocases expressed in *E. coli* was assessed by monitoring the export of a hybrid precursor composed of the RR-signal peptide of MdoD fused to the green fluorescent protein (GFP) as previously described [52]. For these experiments, TatAyCy was expressed from pEXT22 and MdoD-GFP from pBAD24. Cell fractionation into cytoplasmic, periplasmic and membrane fractions was performed as described [52]. The presence of GFP in the different fractions was monitored by Western blotting and immunodetection.

Western blotting and Immunodetection.

The presence of YwbN-Myc, TatAy, LipA and GroEL proteins in cellular and growth medium fractions of *B. subtilis* was monitored by Western blotting. Protein samples for SDS-PAGE were prepared with loading buffer containing reducing agent (Invitrogen). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) by semi-dry blotting (1 hour and 15 min; 1 mA cm^{-2}). For detection of YwbN-Myc, monoclonal Myc-specific antibodies from Gentaur were used. TatAy, LipA, and GroEL were detected using specific polyclonal antibodies (Eurogentec). Antibody detection was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Densitometric image analysis to quantify relative protein amounts as detected by Western blotting was performed with the program ImageJ (http://rsbweb.nih.gov/ij/).

The presence of TatAy and GFP in subcellular fractions of *E. coli* was monitored by SDS-PAGE and Western blotting using specific polyclonal antibodies against TatAy (Eurogentec) and GFP (Promega, Living Colors). Bound antibodies were visualized with goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate and the ECL detection kit (Amersham Pharmacia Biotech). Likewise, the presence of TatCy-Strep-II was detected by Western blotting using a Streptactin-HRP conjugate (Institut fur Bioanalytik) and the ECL detection kit.

Growth experiments.

Strains were pre-cultured in LB medium containing 1% NaCl and subsequently diluted in LB medium with 0% or 1% NaCl to an optical density at 600 nm (OD_{600}) of ~0.01. Growth was continued in a black optical bottom 96-well microtiter plate (Nunc) that was incubated in a Biotek Synergy 2 plate reader (37°C, variable

shaking). OD_{600} readings were recorded for 15 hours.

Expression and purification of TatAyCy and TatAy complexes.

E. coli Δ*tatABCDE* cells containing the mutant *tatAyCy* operons on the pBAD24 plasmid were grown aerobically to mid-exponential phase with induction of the *tatAyCy* genes using 0.5 mM arabinose. Cells were fractionated into membrane and cytosolic components as described previously, and the membranes were solubilised in 2% digitonin [33]. To analyse TatAyCy complexes, the solubilised membranes were incubated with 2 μg/ml avidin to block any biotin-containing proteins before application to an equilibrated 4 ml Streptactin affinity column (Institut fur Bioanalytik). The column was washed with 8 column volumes of equilibration buffer containing Tris-HCl pH 8.0, 2% glycerol, 150 mM NaCl, and 0.1% digitonin. Bound protein was eluted from the column in 6 x 2.0 ml fractions using the same buffer as above, but containing 3 mM desthiobiotin (Sigma). For gel filtration experiments, affinity purified TatAyCy was concentrated to 250 μl using Vivaspin-4 centrifugal concentrators (molecular weight cut off 10,000; Vivascience). The concentrated sample was loaded onto a Superose-6HR gel filtration column (Amersham Biosciences) and was eluted with the equilibration buffer described above [33]. To analyze TatAy complexes, Streptactin column flow-through and wash fractions containing solubilized TatAy complexes were also subjected to gel filtration chromatography and peak elution fractions (18-33) were immunoblotted with anti-TatAy antibodies.

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Chapter 8

Summary, general discussion and perspectives

SUMMARY AND GENERAL DISCUSSION

Due to the very important and diverse functions of the proteins that are exported from the cytoplasm into the cytoplasmic membrane and beyond, bacterial cells cannot survive without effective systems for protein transport. In fact, different protein transport routes have evolved, each serving specific purposes in directing different classes of proteins to different locations in or outside the cell. Protein transport to extracellular locations is generally referred to as secretion. This process of protein secretion is both of fundamental scientific and applied interest, because many of the respective mechanisms are yet to be elucidated and a multitude of biotechnological applications is foreseen for a wide range of potential products.

The Gram-positive bacterium *B. subtilis* has an innate high capacity for the secretion of proteins. For its growth and survival in the soil, it secretes degradative enzymes that liberate nutrients from biomacromolecules, which cannot be taken up directly. Because of these traits *B. subtilis* is extensively used as a Cell Factory for the high-level production of commercially relevant enzymes. In particular, this quality has been exploited by the biotechnology industry as it provides an easy way to obtain high yields of relatively pure protein. In practice, this works particularly well for secretory proteins derived from *B. subtilis* or closely related species. However, the secretion of proteins originating from other prokaryotic or eukaryotic organisms has turned out to be more complicated. Often the yields of such heterologous proteins are unsatisfactory. To find innovative solutions for such applied problems, there is a high need for fundamental research on protein secretion.

As outlined in **chapter 1** of this thesis, the main pathway that is currently exploited for protein secretion is the Sec pathway, which threads proteins through the membrane in an unfolded state. This property probably allows the Sec pathway to translocate many different proteins in large amounts. However, it also sets clear limits to the proteins that can be exported via Sec, such as proteins that assemble a cofactor prior to export from the cytoplasm, or proteins that rapidly fold in the cytoplasm prior to the initiation of their membrane translocation. Thus, the Tat pathway could provide a most welcome alternative solution to these problems since it has a clear bias towards the transport of folded proteins. Importantly, the proteins destined for extracytoplasmic locations are directed to specific transport routes by their N-terminal signal peptides. This means that proteins can in principle be redirected from one route (*e.g.* Sec) to another (*e.g.* Tat) by replacing the original signal peptide with a

signal peptide that directs the proteins into the alternative route. In the case of desired Tat-specific protein transport, this would require provision of the secreted protein with a twin-arginine (RR-)signal peptide. Unfortunately, targeting of Sec-incompatible proteins to the Tat pathway has turned out not as straightforward as initially expected. Frequently, these proteins are still targeted to the Sec pathway or are not secreted at all (**chapter 2**).

At the start of the present PhD research, little information was available concerning the possibilities and limitations for biotechnological exploitation of the "minimal Tat pathways" in *B. subtilis*, their biological function under different growth conditions, and relevant structure-function relationships in the different Tat proteins. Therefore, the work described in this thesis was aimed at a functional analysis of the Tat pathways present in *B. subtilis*.

Two parallel Tat pathways with apparently distinct specificities have previously been discovered in *B. subtilis.* These have become known as TatAyCy and TatAdCd. To explore the application potential of these Tat pathways, several commercially relevant or heterologous model proteins were fused to the signal peptides of the two known *B. subtilis* Tat substrates YwbN and PhoD, which are known to be secreted in strict dependence on TatAyCy or TatAdCd, respectively (**chapter 2**). Indeed the results showed that signal peptide replacement can redirect a protein from the Sec pathway into the Tat pathway as was shown by the YwbN signal peptide-directed secretion of subtilisin, a typical Sec substrate. Specifically, the YwbN signal peptide directed subtilisin secretion via the *B. subtilis* TatAyCy route. By contrast, the same signal peptide directed Tat-independent secretion of the *Bacillus licheniformis* α-amylase (AmyL). Moreover, the YwbN signal peptide directed secretion of SufI, an *Escherichia coli* Tat substrate, in a Tat-independent manner, most likely via Sec. These findings point out two possible bottlenecks for the application of the *B. subtilis* Tat pathways. Firstly, it seems that avoidance of the Sec pathway by engineered Tat substrates is often ineffective. This relates to a lack of understanding how Sec avoidance is regulated in *B. subtilis* at the level of signal peptide function. Secondly, ineffective control of cytoplasmic folding may set limits to the Tat-dependent translocation of engineered precursor proteins. As suggested by the data in chapter 2, cytoplasmic precursor folding is a likely prerequisite for Tat-dependent protein translocation in *B. subtilis*, as is the case in *E. coli*. It thus seems that future applications of the Tat system of *B. subtilis* will, most likely, involve commercially interesting proteins that are Sec-incompatible. This will at least require a better understanding of Sec

avoidance mechanisms and cytoplasmic precursor folding.

In order to be Sec-compatible, precursor proteins need to be in an unfolded state, whereas Tat-compatible proteins need to fold prior to transport. This suggested that the two types of pathways would each export their own specific sets of proteins. Nevertheless, an intriguing early observation from the work on Tat in *B. subtilis* was that many Tat-independently secreted proteins in *B. subtilis* contained potential RR-motifs in their signal peptides. For example, this was the case for the esterase LipA. In the studies described in **chapter 3**, a proteogenomics approach was applied to investigate the secretion mechanism of the esterase LipA. To this purpose, a serendipitously obtained hyper-producing strain was used. The results showed that while LipA was secreted Sec-dependently under standard conditions, hyper-produced LipA was secreted predominantly Tat-dependently via an unprecedented overflow mechanism. In addition, the results obtained with hyperproduced LipA provided first evidence that the two Tat translocases of *B. subtilis* (TatAdCd and TatAyCy) can cooperate in the transport of certain proteins. While the two previously identified *B. subtilis* Tat substrates, PhoD and YwbN, each require a distinct Tat translocase for secretion, the high-level secretion of LipA required the presence of both the TatAdCd and TatAyCy translocases. This shows that these translocases have distinct but overlapping specificities. Importantly, the identified overflow secretion mechanism for LipA focuses interest on the possibility that secretion pathway choice can be determined by environmental and intracellular conditions. This would suggest that at least some of the Sec-dependently transported proteins with potential twin-arginine signal peptides may be exported via the Tat pathway under particular environmental conditions.

One environmental condition that has been implicated in Tat-dependent protein transport is salinity. In recent years, clear evidence has been provided that Tat is of major importance for protein secretion by halophilic archaea (2, 4, 5, 9, 10). Thus, salinity could be a relevant determinant in secretion pathway usage by particular secretory proteins. This raised the question to what extent the growth medium salinity would impact on Tat-dependent protein secretion by *B. subtilis*? The answer to this question is given in **chapter 4**. *B. subtilis tat* mutant strains and the parental strain 168 were grown in medium with different salt concentrations and protein secretion was assessed. Indeed, a major impact of the salinity on the specificity and usage of the *Bacillus* Tat pathway for protein secretion was observed. Experiments performed at

high salinity growth medium conditions revealed clear changes in the specificity of the Tat pathway for the known Tat substrate protein YwbN and they allowed identification of the quality control protease WprA as a new potential Tat substrate. Conversely, experiments with cells grown at low salinity identified the cell wall hydrolase LytD as a new potential Tat substrate. These findings imply that environmental salinity can somehow impact on the cytoplasmic folding of certain proteins such as YwbN, WprA and LytD, directing them either into the Sec or the Tat routes for export. Additionally, salinity may also influence the activity or specificity of the Tat complexes. Interestingly, the studies in **chapter 4** also revealed a clear role of the TatAyCy pathway in iron acquisition. The results indicate that the Dyp-type peroxidise YwbN is required for the acquisition or generation of Fe^{3+}, which is then taken up by the YwbLM high-affinity iron transport system. The importance of this TatAyCy-dependent iron acquisition system is underscored by the finding that it is required for optimal growth and stationary phase survival under conditions of low salinity, and for optimal growth under iron-limited conditions. Overall these data support the view that the *B. subtilis* Tat system has a very important role in iron acquisition.

High resolution or structural information on TatAC-type Tat systems is currently still lacking and the precise signal peptide recognition sites remain yet to be determined. Since it turned out impossible to overproduce the two TatAC complexes in sufficient quantities in *B. subtilis*, the TatAdCd and TatAyCy complexes were overproduced in *E. coli*. As described in **chapter 5**, this allowed analyses on their substrate specificities and complex formation in the context of a Tat-deleted *E. coli* strain. Interestingly, both TatAC complexes were shown to recognize similar signal peptide determinants, which is consistent with the finding that some proteins, like WprA and hyperproduced LipA, require both TatAdCd and TatAyCy for Tat-dependent secretion. Specifically, it was shown that the green fluorescent protein GFP fused to three distinct *E. coli* RR-signal peptides, namely those of AmiA, DmsA and MdoD were translocated both by TatAdCd or TatAyCy when expressed in *E. coli*. Furthermore, site-specific mutagenesis of the DmsA signal peptide confirmed the view that both TatAC translocases of *B. subtilis* recognise similar structural determinants within RR-signal peptides. Nevertheless, also in *E. coli* the specificities of TatAdCd and TatAyCy were not identical as was shown by the finding that the *E. coli* TMAO reductase was translocated by TatAdCd, but not by TatAyCy. Taken together, these findings show that the two TatAC systems of *B. subtilis* are not predisposed to recognize

only specific Tat signal peptides, as was initially suggested by their apparently narrow substrate specificities in *B. subtilis* when cells are grown under standard laboratory conditions. Gel filtration analysis of digitonin-solubilized TatAyCy complexes extracted revealed a discrete ~200 kDa TatAyCy complex that is much smaller than the TatABC complex from *E. coli* (~600 kDa; (8)) or even the TatAdCd complex (~350 kDa; (1)). Also, membrane-bound TatAy complexes were shown to be small (~200 kDa) and homogeneous, which is very different from the situation encountered for *E. coli* TatA which forms membrane-bound complexes varying in size from less than 100 kDa to well over 500 kDa (3, 7). The TatAy complexes were even smaller than the TatAd complexes (~270 kDa; (1)). Taken together, these findings showed that, in particular, the *B. subtilis* TatAd and TatAy complexes differ significantly from the corresponding *E. coli* TatA complexes, which points to major structural differences between Tat complexes from Gram-negative and Gram-positive organisms. Like TatAd, TatAy was also detectable in the form of massive cytosolic complexes of 5 MDa or larger that have previously been implicated in precursor targeting.

A remarkable conclusion from the experiments presented in **chapter 5** was that the *B. subtilis* TatAC translocases were both able to translocate active GFP when expressed in *E. coli*. In contrast, earlier experiments had indicated that this was not possible in *B. subtilis* (**chapter 2**; (6)). It was therefore of interest to assess whether the same RR-signal peptide-GFP hybrid precursors that were Tat-dependently translocated in *E. coli* would also be exported Tat-dependently in *B. subtilis*. The results of these studies are described in **chapter 6**. Despite their effective translocation by heterologously expressed TatAdCd or TatAyCy complexes in *E. coli* (**chapter 5**), the GFP fusion proteins with signal peptides of AmiA, DmsA and MdoD were not Tat-dependently secreted when expressed in *B. subtilis*. Also unlike the situation in *E. coli*, Tat-independent GFP secretion was demonstrated. Notably, at high salinity growth conditions, the Tat-independent secretion of GFP was generally enhanced and this effect was strongest in strains lacking TatAyCy. In this respect, the most drastic effects were observed with the DmsA-GFP hybrid for which no secretion was detectable when cells were grown in LB medium with 1% salt, but for which secretion was clearly detectable when cells were grown in 6% salt. Coincidentally, of the three tested hybrid GFP precursors, the DmsA-GFP precursor happens to be the only one that contains a positively charged residue (R) in the c-terminal part of the signal peptide, which might thus have a potentially active role in Sec avoidance in

B. subtilis. Taken together, these results indicate that as yet unidentified quality control mechanisms reject the GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to their Sec-dependent secretion. At least the Sec avoidance of all three hybrid GFP precursors seems to be overruled when cells are grown in medium with 6% salt. Furthermore, at least under these high salinity growth conditions, the TatAyCy translocase seems to be a determinant in Sec avoidance. Most likely, the identification and subsequent elimination or modulation of the quality control systems that determine GFP secretion will be key to unlocking the *B. subtilis* Tat pathway for the production of heterologous proteins.

One feature that adds flavour to studies on Gram-positive bacterial TatAC systems, as encountered in *B. subtilis*, is the absence of the TatB component that it is present in the TatABC translocases in Gram-negative bacteria and chloroplasts. This implies that the TatA component of the TatAC systems is bifunctional in the sense that it performs both the roles of TatA and TatB in TatABC translocases. This view was recently confirmed by the heterologous complementation of *E. coli tatA* or *tatB* mutants with the *B. subtilis* TatAd protein (1). This focused interest on residues that are important for *B. subtilis* TatA function.

Chapter 7 describes a site-directed mutagenesis study performed with TatAy of *B. subtilis*. Interestingly, one amino acid substitution in TatAy (*i.e.* F19A) led to significantly improved secretion of YwbN. By contrast, several amino acid substitutions were shown to eliminate TatAy function in the secretion of YwbN, at least when cells were grown in LB medium with 1% salt. The respective residues in the authentic TatAy were thus identified as being critical for TatAy stability, complex formation and/or function. With only one exception (*i.e.* TatAy P2D), the mutant TatAy proteins were shown to form complexes with TatCy. Most of these complexes resembled the wild-type TatAyCy complexes in size, but a few TatAy mutant protens (*i.e.* TatAy K22A and P25A) seemed to form larger complexes with TatCy. In particular, the formation of "TatAy only" complexes appeared to be affected by the different mutations, all inactive mutants showing TatAy complexes that were much more heterogeneous in size than the wild-type TatAy complex. This was particularly evident for the P2A mutant protein. The effects on TatAy complex formation may at least in part explain why the respective mutant TatAy proteins did no longer facilitate effective YwbN secretion when cells were grown in LB with 1% salt. Importantly, however, the activity of the mutant TatAy proteins was also tested by growing cells in LB medium without added

salt. This revealed that nearly all of the mutant TatAy proteins had residual activity under these conditions, indicating that conditional salt-sensitive Tat translocases had been created. This would suggest that TatAC translocases could be intrinsically salt-sensitive and that this effect was perhaps enhanced in the constructed TatAy mutants. To test this idea, TatAC complexes from *Bacillus cereus* and *Staphylococus aureus* were expressed in *B. subtilis*. These TatAC complexes had low or no activity with respect to YwbN secretion when cells were grown in medium with 1% NaCl. By contrast, the YwbN translocating activity of the *B. cereus* TatAC was very strongly enhanced when cells were grown at 0% NaCl, and for TatAC of *S. aureus* YwbN translocating activity was clearly detectable under these conditions. Consistent with this view, both the *B. cereus* and *S. aureus tatAC* genes complemented the growth defects of *tatAyCy* mutant *B. subtilis* strains grown in LB medium with no added salt. It thus seems that at least the TatAC translocases from Firmicutes such as *B. cereus* and *S. aureus* are intrinsically salt-sensitive. This would suggest that ionic interactions play an important role in TatAC function.

FUTURE PERSPECTIVES

Taken together, the results described in this thesis provide novel insights in different aspects of minimal TatAC pathway function in *B. subtilis* that may hold the key to unlocking this pathway for biotechnological purposes, such as the secretion of novel classes of industrial enzymes or pharmaceutically relevant proteins. It can be foreseen that this will require an integrated approach that focuses on at least three relevant aspects, namely: 1. identification of optimal RR-signal peptides; 2. removal or modulation of quality control systems that result in the rejection of proteins by Tat; and 3. enhanced Sec avoidance. As to the development of optimal signal peptides, the results in this thesis suggest that the YwbN signal peptide might be a useful starting point since it was successfully used for the Tat-dependent secretion of subtilisin. On the other hand, the *E. coli* DmsA signal peptide appears attractive, because it was the only one that facilitated effective Sec avoidance, at least when fused to GFP and expressed in cells that were grown in LB with 1% salt. For the removal or modulation of quality control systems, the work in this thesis has not yet provided a clear solution, but at least interesting tools for this work are now available in the form of hybrid GFP precursors that have the intrinsic potential for TatAdCd and TatAyCy-dependent translocation. These

precursors might allow the development of sensitive assays allowing the identification of mutant *B. subtilis* strains that secrete active GFP. The best way to enhance Sec avoidance will probably lie in optimising the cytoplasmic conditions for protein folding in combination with the use of signal peptides that are poorly recognized by the Sec machinery.

With respect to fundamental research on minimal Tat function, the present studies have raised a number of intriguing questions that remain to be answered. These concern for example the folding state of proteins that are secreted via the *B. subtilis* TatAC pathways. Although it seems most likely that the proteins are transported in a folded state, it is known from work in *E. coli* and chloroplasts that the Tat system can also handle a limited number of unfolded substrates. Whether this is also true for the minimal Tat systems in *B. subtilis* remains to be demonstrated. Likewise, it will be important to investigate whether or how LipA hyperproduction influences the folding state of the LipA precursor, or how environmental salinity impacts on the folding states of the YwbN, WprA or LytD precursors thereby allowing or precluding Tat-dependent secretion. Especially with respect to the effects of environmental salinity, it will be important to investigate how exactly this parameter impacts on TatAC complex formation, translocation activity, cooperativity of the TatAdCd and TatAyCy translocases or Sec avoidance. This may help to answer the question why at least some TatAC translocases appear intrinsically salt-sensitive.

Lastly, it will be interesting to determine which structural features in TatAd or TatAy determine the bifunctionality of these proteins, allowing them to perform the functions of both the TatA and TatB subunits of the TatABC translocases. Many of these questions can probably be answered at least in part by structural analyses of TatAC complexes. To this end, the TatAyCy complex seems an ideal candidate since it is the smallest homogeneously sized Tat complex known to date, as shown in chapter 5 of this thesis.

In conclusion, it is clearly evident from the present PhD studies that many interesting questions concerning Tat-dependent secretion in *B. subtilis* remain to be answered. On the other hand, very significant progress has also been made and interesting leads have been provided to answer the open questions. Thus, this work will hopefully form a good starting point for sorting out the rules that govern protein transport via the minimal Tat pathways of *B. subtilis*.

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Nederlandse samenvatting

Transport van gevouwen eiwitten via het *Bacillus subtilis* Tat systeem

Samenvatting

Bacteriën gebruiken specifieke transportsystemen voor het opnemen en uitscheiden van verschillende moleculen. Deze getransporteerde moleculen hebben ondermeer functies in de bacteriële stofwisseling, de verdediging van bacteriën tegen andere organismen en de communicatie tussen verschillende bacteriële cellen. Enkele transportsystemen zorgen specifiek voor de uitscheiding van eiwitten (secretie). Onderzoek naar eiwitsecretie in Gram-positieve bacteriën wordt bij voorkeur gedaan met het modelorganisme *Bacillus subtilis*. De hoge capaciteit voor eiwitsecretie maakt deze bacterie toepasbaar in de biotechnologische industrie, als "cellulaire fabriek" voor waardevolle gesecreteerde eiwitten. Dit geldt voornamelijk voor soorteigen eiwitten. Secretie van soortvreemde eiwitten gaat vaak moeizaam. Nieuwe inzichten zijn daarom nodig om de toepasbaarheid van *B. subtilis* te vergroten. De studies in dit proefschrift zijn gericht op een systeem voor de secretie van gevouwen eiwitten. Deze eiwitten bezitten een moleculaire postcode, het signaalpeptide, met twee arginine residuen, waaraan het systeem zijn naam ontleent: de "Twin-arginine translocase" ofwel Tat. Het TatAC systeem van *B. subtilis* vertegenwoordigt een minimale variant van dit systeem, omdat slechts twee componenten nodig zijn voor activiteit. Het beschreven onderzoek laat zien, dat het mogelijk is om eiwitten specifiek via het Tat systeem van *B. subtilis* te laten secreteren. Een belangrijk nieuw inzicht is dat interne en externe factoren van invloed zijn op het gebruik van Tat. Zo blijkt sterke overproductie van de esterase LipA in secretie via Tat te resulteren. Tevens blijkt de zoutconcentratie in het groeimedium van belang voor Tat-afhankelijke eiwitsecretie. Dit ligt waarschijnlijk aan een intrinsieke gevoeligheid van het Tat systeem voor zout. De studies geven inzicht in functie en toepassingsmogelijkheden van het *Bacillus* Tat systeem.

BACTERIËN

Bacteriën zijn eencellige micro-organismen, die overal op deze planeet leven. Dit betekent dat bacteriën voorkomen op heel gewone plekken zoals op je huid en haar, op het aanrecht in de keuken en in je voeding, maar ook op extreme en misschien minder verwachte plekken zoals diepe oceaanbodems of hete geiserbronnen op IJsland. Met een grootte van één of meerdere micrometers (een duizendste van een millimeter) zijn bacteriën de kleinste levende organismen. De vorm is afhankelijk van de soort. Zo kunnen er ronde, ovale, spiraalvormige en staafvormige bacteriën onderscheiden worden. De vele verschillende soorten bacteriën, die we op dit moment kennen (we kennen ze nog niet allemaal), kunnen onderverdeeld worden in Gram-negatieve en Gram-positieve bacteriën. De classificatie Gram-positief en Gram–negatief is ontstaan, doordat de ene groep bacteriën (de Gram-positieven) een kleurstof duidelijk beter blijkt vast te houden dan de andere groep (de Gram-negatieven). Dit verschil heeft te maken met verschillen in de samenstelling van de celenvelop van de Gram-positieve en Gram-negatieve bacteriën. De studies in dit proefschrift zijn voornamelijk uitgevoerd met de Gram-positieve bacterie *Bacillus subtilis*, maar in enkele gevallen ook met de Gram-negatieve bacterie *Escherichia coli* (hoofdstuk 5 en deels hoofdstuk 7).

De ongevaarlijke bodembacterie *B. subtilis* heeft verschillende eigenschappen, die hem geschikt maken voor fundamenteel wetenschappelijk onderzoek en voor toepassingen in de biotechnologische industrie. In wetenschappelijk onderzoek wordt deze bacterie als rolmodel gebruikt om meer inzicht te krijgen in de verschillende levensprocessen van Gram-positieve bacteriën. In dit proefschrift is bijvoorbeeld fundamenteel wetenschappelijk onderzoek gedaan naar een mechanisme van eiwituitscheiding door *B. subtilis*. In de biotechnologie is *B. subtilis* belangrijk voor de productie van verschillende, commercieel interessante en toepasbare producten zoals bijvoorbeeld de enzymen, die worden toegevoegd aan wasmiddelen. Deze enzymen zijn eiwitten, die helpen bij het schoonmaken van de was door etensresten of bloed af te breken, zodat vlekken beter verwijderd worden. De mogelijkheden om *B. subtilis* als “cellulaire fabriek” te gebruiken voor soortvreemde “niet eigen” eiwitten zijn helaas beperkt. Dit komt voornamelijk doordat we nog niet volledig begrijpen hoe de verschillende eigenschappen van *B. subtilis* het beste voor dit doel ingezet kunnen worden. Om alle door *B. subtilis* geboden mogelijkheden te benutten dienen we beter te begrijpen hoe we ze kunnen toepassen. De studies in dit proefschrift belichten verschillende aspecten van de secretie van gevouwen

eiwitten met als doel dit proces beter te begrijpen om meer toepassingsmogelijkheden voor *B. subtilis* te creëren.

DNA, EIWITTEN EN DE WERKING VAN DE BACTERIËLE CEL

Een bacterie is opgebouwd uit eiwitten, suikers, vetten en water. De vetlaag die bestaat uit fosfolipiden wordt ook wel de celmembraan genoemd. De celmembraan zorgt voor een afscheiding van het interne waterige milieu en de externe omgeving. Je kunt dit het beste visualiseren als de binnenbal van een leren voetbal, die alle bestanddelen binnen in de bacteriecel bijeenhoudt. Om de membraan zit een stevige celwand, vergelijkbaar met het leer van de voetbal, die de bacterie stevigheid geeft. In de cel en in de celmembraan zitten eiwitten. Deze eiwitten voeren sleutelfuncties uit voor de bacterie. Zij zorgen er voor dat al het voedsel wordt opgenomen, wordt verwerkt en dat afvalstoffen worden uitgescheiden. Binnen in de bacterie zijn nog heel veel andere eiwitten aanwezig met allemaal een eigen functie. Hoe al deze eiwitten gemaakt moeten worden staat beschreven in het DNA. Daarom wordt DNA ook wel de "blauwdruk" van de cel genoemd. Sommige eiwitten in de bacterie lezen in het DNA hoe een specifiek eiwit er uit moet zien en gaan dit samen met andere eiwitten maken. Een stuk DNA waarin staat hoe een eiwit er uit moet zien heet een gen. Een eiwit is opgebouwd uit een keten van aminozuurresiduen. Je kunt je dit voorstellen als een kralenketting met een begin en een eind. Elke kraal vertegenwoordigt dan een aminozuurresidu. De verschillende combinaties en lengtes zorgen er voor, dat elk eiwit er anders uit ziet. Sommige residuen in zo'n kralenketting hebben delen, die niet of juist wel in contact willen komen met water. Wanneer het eiwit geproduceerd is gaan alle "watervrezende" (hydrofobe) delen zich naar binnen richten en de "waterminnende" (hydrofiele) delen naar buiten waardoor ze met water in contact komen. Dit proces van eiwitvouwing is zo complex, dat men op basis van de aminozuurvolgorde van een eiwit nog niet kan voorspellen hoe het proces verloopt en hoe de structuur er uitziet.

Om het DNA, de eiwitten en vetten te kunnen maken hebben bacteriën bouwstoffen nodig zoals bijvoorbeeld koolhydraten (suikers), aminozuren en water. Ook zijn vitamines en metalen van belang zoals magnesium, ijzer, koper, calcium, kalium en natrium. Deze voedingsstoffen worden continu opgenomen om de bacterie in staat te stellen om te blijven groeien en delen.

Tevens worden afvalstoffen steeds uitgescheiden. Zo lang er voldoende voedingsstoffen zijn is een bacterie continu bezig met groeien en zich zelf te vermenigvuldigen. Om een vitaal nageslacht te kunnen voortbrengen dient de moedercel al het essentiële materiaal (DNA, eiwitten en vetten) door te geven aan de dochtercellen. De voortplanting van de cel verloopt door middel van deling. Dit betekent dat in principe elke cel deelt. Als je begint met 1 cel krijg je er vervolgens 2, dan 4, dan 8, 16, 32 etc. Hierdoor neemt het aantal cellen tijdens de groei exponentieel toe. In principe heb je daarom maar één bacteriecel nodig om oneindig veel dochtercellen te kunnen maken. De celdeling stopt meestal of komt op een "steady state" (dit betekent dat er evenveel cellen dood gaan als er bij komen) wanneer het voedsel op raakt of wanneer er te veel toxische stoffen aanwezig zijn in het milieu, waardoor er veel cellen dood gaan.

EIWITTRANSPORT

In groeiende cellen is er een continue aanmaak van eiwitten, verdubbeling van DNA en productie van extra fosfolipiden voor de celmembraan. De mens heeft geleerd gebruik te maken van deze eigenschappen van de bacterie. Naast het essentiële "blauwdruk" DNA zijn er kleine circulaire DNA-fragmenten gevonden, plasmides genaamd. Deze plasmides zijn niet van nature in elke bacterie aanwezig en ze zijn in principe niet nodig voor het overleven van een bacterie. Plasmides dragen echter soms extra genen, die onder specifieke condities belangrijk worden om te overleven. Dit geldt bijvoorbeeld voor genen die zorgen voor antibioticumresistentie van de bacterie. Onder normale groeicondities zijn plasmides met dergelijke genen niet belangrijk, maar wanneer de bacterie in aanraking komt met een antibioticum dan heeft hij een resistentiegen nodig om bestand te zijn tegen dit antibioticum. Met moleculair biologische technieken heeft men geleerd om het plasmide DNA te isoleren uit de cel en het vervolgens te modificeren, door bijvoorbeeld extra genen toe te voegen. Als dit plasmide met extra genen nu weer terug gebracht wordt in de cel, leest de cel deze extra genen af, hetgeen zal leiden tot de aanmaak van de nieuwe extra eiwitten.

De vele verschillende eiwitten in een bacterie hebben allemaal eigen functies. Sommige eiwitten hebben een functie in de cel en andere in de membraan. Er zijn echter ook eiwitten die belangrijke taken uitvoeren buiten de celmembraan, bijvoorbeeld in de celwand of in het externe

milieu. Eiwitten die gesecreteerd worden in het externe milieu dienen bijvoorbeeld voor het beschikbaar maken of verteren van voedsel, dat dan vervolgens door de bacterie opgenomen kan worden. Om die eiwitten buiten de cel te krijgen worden er door de cel transportsystemen gemaakt (die zelf ook weer uit eiwitten bestaan), die de desbetreffende eiwitten door de membraan brengen zonder de membraanbarrière te verbreken. Als deze barrière verbroken zou worden dan zou er een gat ontstaan, waardoor de bacterie zijn inhoud van eiwitten, DNA en moeizaam vergaarde voedingstoffen zou verliezen. Net als bij een voetbal, is de druk binnen de bacteriecel groter dan buiten de cel en loopt hij leeg wanneer er een lek in het omhulsel zit. De eiwittransportsystemen hebben dus een uiterst belangrijke functie. Deze transportsystemen zitten in de membraan van de cel waar ze een poortfunctie vervullen. Eiwitten die een functie buiten de cel hebben moeten specifiek door een transportsysteem herkend kunnen worden, anders weet de bacterie niet dat het eiwit naar buiten moet. Een specifieke herkenning en sortering van uit te scheiden eiwitten is van belang omdat er meerdere verschillende transportsystemen zijn om eiwitten met verschillende eigenschappen te secreteren. Het ene systeem kan vaak niet de eiwitten van een ander systeem naar buiten brengen. Voor de specifieke herkenning van extracellulaire eiwitten door eiwittransportsystemen wordt een label, het zogenaamde signaalpeptide, aan het begin van het eiwit geplaatst, al tijdens de synthese van dit eiwit. Signaalpeptides bestaan uit complexe en specifieke codes van verschillende aminozuren (je kunt ze beschouwen als postcodes voor kaarten of brieven). Soms is het verschil tussen deze signaalpeptides klein en is het moeilijk voor wetenschappers te voorspellen welk systeem door de bacterie gebruikt zal worden om het eiwit te secreteren.

In dit proefschrift beschrijf ik resultaten van onderzoek aan een transportsysteem, dat eiwitten naar buiten brengt in hun uiteindelijke vorm, dus in de volledig gevouwen toestand. Dit is een unieke eigenschap van dit transportsysteem, die voor de biotechnologische industrie interessante toepassingsmogelijkheden zou kunnen bieden om soortvreemde eiwitten door bacteriën te laten uitscheiden. Het bestudeerde transportsysteem heet de "Twin-arginine translocase" of in het kort Tat. Deze naam heeft het transportsysteem gekregen omdat het eiwitten over de celmembraan transporteert, die signaalpeptides hebben met twee naast elkaar gelegen arginine aminozuurresiduen. In bacteriën is het Tat transportsysteem als eerste ontdekt in de darmbacterie *E. coli* en pas later in *B. subtilis*. De bacteriën hebben beide een verschillend Tat systeem. Vandaar dat dit proefschrift naast de studies

aan het Tat systeem in *B. subtilis* ook studies aan het Tat systeem in *E. coli* beschrijft. Het minder bekende *B. subtilis* systeem, dat ik samen met verschillende collega's bestudeerd heb, is opgebouwd uit de twee eiwitten TatA en TatC. Daarom wordt dit systeem vaak een TatAC systeem genoemd. In *B. subtilis* zijn zelfs twee verschillende types TatAC systemen aanwezig. Deze worden TatAyCy en TatAdCd genoemd. Helaas weten we nog niet voldoende van deze transportsystemen om ze gericht en met succes toe te passen voor biotechnologische doeleinden. Dit heeft wellicht te maken met het feit, dat niet compleet duidelijk is onder welke leefomstandigheden van de bacterie het transportsysteem belangrijk is. In ons onderzoek hebben we ons daarom in eerste instantie gericht op het signaalpeptide aan het begin van het te transporteren eiwit, om beter te kunnen begrijpen wat er nu voor zorgt dat het eiwit gebruik maakt van het Tat transportsysteem. Verder hebben we onderzocht hoeveel eiwitten getransporteerd worden via dit systeem. Om beter te begrijpen hoe het transportsysteem werkt hebben we ook hieraan onderzoek gedaan. Tevens hebben we bekeken of het transportsysteem anders werkt wanneer de zoutconcentraties in de leefomgeving van *B. subtilis* veranderen. Dit is namelijk een situatie die de *B. subtilis* cel veelvuldig tegenkomt in zijn natuurlijke leefomgeving. Onze modelbacterie *B. subtilis* leeft namelijk in de bodem en tussen de wortels van planten.

HET ONDERZOEK EN DE RESULTATEN

Een algemene inleiding over eiwittransport is gegeven in **hoofdstuk 1** van dit proefschrift, waarbij de nadruk is gelegd op eiwittransport via het Tat systeem.

In **hoofdstuk 2** is onderzocht of twee signaalpeptides van eiwitten, die gesecreteerd worden via het *B. subtilis* Tat systeem, ook andere "niet-Tat" eiwitten naar het Tat systeem kunnen dirigeren voor secretie via dit systeem. Duidelijk is gebleken dat eiwitten, die in *B. subtilis* niet via Tat gesecreteerd worden, niet zomaar door het Tat systeem geaccepteerd worden wanneer ze een Tat signaalpeptide krijgen. Één van de eisen lijkt te zijn, dat ze hun uiteindelijke gevouwen structuur moeten hebben om getransporteerd te kunnen worden.

Hoofdstuk 3 beschrijft het reddende effect van het Tat systeem wanneer een ander transportsysteem één van zijn eiwitten, genaamd LipA, niet snel genoeg of in voldoende mate naar buiten kan brengen. Het Tat systeem accepteert onder deze "noodcondities" het LipA eiwit voor transport, terwijl dit anders nauwelijks

detecteerbaar gebeurt. Het betekent ook, dat LipA onder deze omstandigheden via twee verschillende transportsystemen het externe milieu bereikt. In de bodem, de natuurlijke omgeving van *B. subtilis*, kan het voorkomen dat de zoutconcentratie toeneemt of afneemt, bijvoorbeeld door verdamping van water of regenval. Om te onderzoeken of de cel meer of minder gebruik maakt van het Tat systeem onder deze verschillende condities hebben we *B. subtilis* bij verschillende zoutconcentraties gekweekt en de Tat-afhankelijke secretie van eiwitten geanalyseerd. De resultaten zijn beschreven in **hoofdstuk 4**. Diverse extracellulaire eiwitten blijken, afhankelijk van de zoutconcentratie in het groeimedium, ook gebruik te kunnen maken van het Tat systeem, terwijl ze onder de standaard laboratoriumcondities via een ander transportsysteem gesecreteerd worden. Tevens blijkt het Tat systeem belangrijk voor normale groei in de afwezigheid van zout. Onder deze omstandigheden heeft de afwezigheid van het Tat systeem een abnormale periode van celsterfte tot gevolg. Dit kan voorkomen worden door de *tat* genen weer terug te brengen in de bacterie met behulp van een plasmide of door extra ijzer aan de cel aan te bieden. Bij afwezigheid van zout in the groeimedium blijkt het Tat systeem namelijk indirect belangrijk te zijn voor de opname van ijzer. Verder blijkt uit de resultaten, dat voor de secretie van sommige eiwitten beide Tat systemen (TatAyCy en TatAdCd) van *B. subtilis* belangrijk zijn.

Het minimale Tat transportsysteem van *B. subtilis* is opgebouwd uit twee verschillende eiwitten, TatA en TatC. Deze eiwitten zitten in de membraan en vormen in meerdere kopieën samen een transportsysteem. De grootte van het TatAyCy systeem was nog onbekend, hetgeen de aanleiding was voor het onderzoek dat beschreven is in hoofdstuk 5. Het TatAyCy systeem blijkt kleiner te zijn dan het TatAdCd systeem van *B. subtilis*. Verder herkennen beide transportsystemen dezelfde aminozuurresiduen in het signaalpeptide van het te transporteren eiwit. Hoewel TatAyCy en TatAdCd in veel opzichten op elkaar lijken blijkt dat er verschillen zijn in het spectrum van eiwitten die deze systemen kunnen transporteren.

In **hoofdstuk 5** is gebruik gemaakt van een fluorescent eiwit, het "Green Fluorescent Protein" ofwel GFP. Dit fluorescerende eiwit kent veel toepassingen in het fundamenteel wetenschappelijke onderzoek. Als GFP in de cel gemaakt wordt en goed vouwt is dat te detecteren door de afgifte van groen licht. Door het GFP eiwit te koppelen aan verschillende signaalpeptiden hebben we onderzocht onder welke condities het geaccepteerd zou kunnen worden door het TatAyCy transport systeem. Dit onderzoek is in eerste instantie

uitgevoerd in *E. coli* door de genen voor het TatAyCy systeem in deze bacterie te brengen en vervolgens de functie van het systeem experimenteel te toetsen. Om te bekijken of het TatAyCy systeem zich op dezelfde manier gedraagt in *B. subtilis* is een deel van het onderzoek in hoofdstuk 5 herhaald met deze bacterie. Dit werk staat beschreven in **hoofdstuk 6**. Het onderzoek aan de secretie van GFP hebben we verder aangevuld door de invloed van een verhoogde externe zoutconcentratie te analyseren. Hierbij is gebleken dat eigenschappen, die onder standaard laboratoriumcondities verhinderen dat een eiwit met een Tat signaalpeptide via een ander systeem gesecreteerd worden, niet meer goed functioneren bij groei in een zout milieu. Het lijkt er op dat het TatAyCy systeem een controle uitvoert op het te transporteren eiwit, bijvoorbeeld of het goed gevouwen is. Als het eiwit niet goed gevouwen is wordt het niet door het Tat systeem getransporteerd. Zo'n kwaliteitscontrole functie is reeds toegekend aan het Tat transportsysteem van de *E. coli* bacterie.

In **hoofdstuk 7** is onderzocht wat de functies van specifieke aminozuurresiduen zijn in het TatAy eiwit met betrekking tot de structuur en functie van TatAy. Het TatAy eiwit is belangrijk voor het Tat transportsysteem, omdat het essentieel is voor de herkenning van het Tat signaal peptide en later in het transportproces hoofdzakelijk het kanaal vormt waardoor het eiwit de membraan passeert. Specifieke aminozuurresiduen in TatAy zijn vervangen met alanine aminozuurresiduen. De TatAy eiwitten met zo'n vervanging worden mutante eiwitten genoemd. De meeste TatAy mutanten kunnen minder goed een functioneel transportsysteem vormen en de veranderde aminozuurresiduen zijn dus belangrijk voor eiwittransport. In een enkel geval blijkt een mutant TatAy eiwit aanleiding te geven tot verbeterd eiwittransport. Deze mutant is wellicht interessant voor toekomstige toepassingen voor de secretie van "soortvreemde" eiwitten. Verder is gebleken dat alle TatAy mutanten "zoutgevoelig" zijn. Dit duidt er op dat de mogelijkheid om een functioneel Tat systeem te vormen niet alleen afhangt van de aminozuur samenstelling van de Tat eiwitten (TatA in dit geval), maar ook van de externe zoutconcentratie. Tat systemen afkomstig van sommige andere Gram-positieve bacteriën blijken ook zoutgevoelig te zijn.

In het laatste hoofdstuk, **hoofdstuk 8** zijn de resultaten en inzichten samengevoegd om er een duidelijke, overzichtelijke en complete samenvatting van te geven. Tevens wordt het onderzoek in een breder perspectief geplaatst.

English summary

Transport of folded proteins via the *Bacillus subtilis* Tat system

LAYMAN'S SUMMARY

Bacteria use specific transport system for uptake and secretion of molecules with functions in metabolism, defence against other organisms and communication between cells. The purpose of some transport systems is the secretion of proteins. Research on protein secretion in Gram-positive bacteria is commonly performed with the model organism *Bacillus subtilis*. The high protein secretion capacity of this bacterium makes it applicable in the biotechnological industry as a "cell factory" for valuable secreted proteins. This accounts mostly for proteins from bacilli. Secretion of proteins from other organisms is often difficult. More research is needed to increase the applicability of *B. subtilis*. The present studies focus on a system that can transport folded proteins. The transported proteins carry a molecular zip-code, the signal peptide, that harbours two neighbouring arginine residues. Therefore the system is named the "Twin arginine translocase", or in short Tat. The TatAC system in *B. subtilis* represents a minimal variant of this system, because only two components are necessary for activity. The results show that it is possible to target proteins for secretion via the *B. subtilis* Tat system. Novel insights are that internal and external factors can influence the use of Tat. High-level overproduction of the esterase LipA results in its secretion via Tat. Also, the salinity of the growth medium is important for Tat-dependent secretion. This is apparently to be due to an intrinsic salt-sensitivity of the Tat system. Overall, this PhD research provides insights in the function and application potential of the *B. subtilis* Tat system.

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MEDISCHE MICROBIOLOGIE / MOLECULAIRE BACTERIOLOGIE

Nederlands

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English

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THE EUROPEAN TAT MACHINE CONSORTIUM

Nederlands

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[#] = both authors contributed equally